# SOME STUDIES ON REPRODUCTIVE PHYSIOLOGY OF THE FEMALE GROUPER, *EPINEPHELUS DIACANTHUS* (VALENCIENNES)

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OF THE REQUIREMENTS FOR THE DEGREE OF

***DOCTOR OF PHILOSOPHY***

IN

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*BY*


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**AUGUST 2007**

*Dedicated To*

*MyFamily...*

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# CERTIFICATE

Certified that the thesis entitled "SOME STUDIES ON REPRODUCTIVE PHYSIOLOGY OF THE FEMALE GROUPER, *EPINEPHELUS DIACANTHUS* (VALENCIENNES)" is a record of independent bonafide research work carried out by Mr. A. Chandrasekhara Rao during the period of study from September 2001 to September 2007 under our supervision and guidance for the degree of Doctor of Philosophy in Fish and Fisheries Science (Mariculture) of the Central Marine Fisheries Research Institute, C.I.F.E (Deemed University), Mumbai, and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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# DECLARATION

I hereby declare that the thesis entitled " **SOME STUDIES ON REPRODUCTIVE PHYSIOLOGY OF THE FEMALE GROUPER, *EPINEPHELUS DIACANTHUS* (VALENCIENNES)**" is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

31 August 2007
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# सारांश

दक्षिण पूर्व एशिया में खूब पालन की जाने वाली प्रचलित मछली है ग्रूपर. पालन केलिए बड़ी शक्यता होने वाली इस मछली जाति की तेज़ बढ़ती, आहार रूपांतरण की क्षमता और उच्च बाज़ार भाव होते है. ग्रूपर मात्स्यिकी की वाणिज्यिक प्रमुख जातियाँ हैं *एपिनिफेलस डयाकान्तस*, *ई. ब्लीकेरी*, *ई. क्लोरोस्टिग्मा*, *ई. मलबारिकस* और *ई. टॉविना*. लेकिन हमारे देश में प्राकृतिक रूप से ग्रूपर मछली बीज मिलना मुशकिल है और स्फुटनशाला में उत्पादित बीजों की भी अपर्याप्तता होती है. इस कारण से बडे पैमाने में ग्रूपर मछली की संवर्धन कार्यविधियों में समस्या उत्पन्न होती है. किसी भी पालन योज्य पख मछली का नियंत्रित अवस्था में प्रजनन और बडे पैमाने में उत्पादन करने केलिए पुनरुत्पादन अवस्था के दौरान मछली के शरीर के अंतरा कोशिक और अंतःकोशिक स्तर के जटिल शरीरक्रिया परिवर्तनों के बारे में समझना अनिवार्य है. परिपक्वता के समय जननग्रंथि में कई विकासात्मक परिवर्तन होते है और साथ साथ कोशिकीय, जैवरासायनिक और आण्विक परिवर्तन भी होते हैं. वर्तमान अध्ययन में कुल समुद्री अवतरण में प्रमुख योगदान करने वाली तलमज्जी मात्स्यिकी की मादा ग्रूपर मछली *ई. डयाकान्तस* के पुनरुत्पादी शरीरक्रिया विज्ञान पर प्रकाश डाला गया है. कोइलोन और रत्नगिरी क्षेत्रों में भारतीय मात्स्यिकी सर्वेक्षण के पोत द्वारा किए गए सर्वेक्षण के दौरान संग्रहित मछली नमूनों पर अध्ययन चलाया गया. अध्ययन में मादा मछली की पुनरुत्पादन अवस्था का विवरण और परिपक्वन दशा I से दशा III तक का वर्गीकरण भी किया गया है. इसके अतिरिक्त जी एस आइ (गोनाडो सोमाटिक इन्डेक्स), एच एस आइ (हेपाटो सोमाटिक इन्डेक्स), सी एफ (कन्डीशन फाक्टर), जननक्षमता आदि के आंकडों का सांख्यिकीय निर्वचन भी किया गया है. अंडजनन के दौरान होने वाले अंतराकोशिक और अंतःकोशिक परिवर्तन समझने के लिए माइक्रोस्कोपिक और अल्ट्रा स्ट्रक्चरल अध्ययन द्वारा अंडक और यकृत के ऊतक - विज्ञान का विवरण दिया गया. विकृतीकरण नहीं किए गए नेटीव और विकृतीकरण किए गए SDS - PAGE द्वारा ओवेरियन होमोजनेट और ब्लड सीरम में होनेवाले विटेल्लोजेनिन का वर्गीकरण किया गया. SDG-PAGE में विटेल्लोजेनिन प्रोटीन खंडों के आण्विक भार का निर्धारण किया गया है. परिपक्वता के दौरान होनेवाले जैव रासायनिक परिवर्तन का समर्थन करने के आंकडे प्राप्त करने केलिए चार विभिन्न ऊतकों के प्रमुख सात प्राचलों पर अध्ययन किया गया. उपर्युक्त पहलुओं पर किए गए अध्ययन से प्राप्त सूचनाओं के आधार पर बडी ग्रूपर मछलियों पर आगे का अध्ययन आसान हो जाएगा.

## ABSTRACT

Groupers are popular food fish farmed in South East Asia and have potential to become an important cultivable species owing to their fast growth, efficient feed conversion and high market price. Commercially important species contributing to the grouper fishery mainly includes *Epinephelus diacanthus E. bleekeri, E. chlorostigma, E. malabaricus* and *E. tauvina.* A major bottleneck in the large scale onshore culture activities of groupers in our country is the lack of adequate number of hatchery produced seed of groupers since wild seed availability is highly fluctuating and unpredictable. In order to master controlled breeding and achieve mass scale production of seed of any cultivable fin fish species, an in depth understanding of the complex physiological changes that occur at intracellular and intercellular level in the fish body during the process of reproduction is inevitable. The gonads with the onset of maturation show a series of developmental changes, which are closely accompanied by the cellular, biochemical, and molecular changes. It is against this background that the present study has been taken up on the reproductive physiology of female grouper *E. diacanthus* which sustain one of the major demersal fisheries of our country. The study was carried out from collections of the fish onboard FSI vessel during the cruises off Quilon and off Ratnagiri region. In this study, the morphology of the female reproductive system has been described in detail followed by the detailed classification of maturity stages from stage I to stage III. Data on GSI (Gonadosomatic Index), HSI (Hepatosomatic Index), CF (Condition Factor), fecundity etc. have also been obtained and statistically interpreted. The detailed histology of oocytes and hepatocytes has been described through microscopic and ultra structural studies to understand inter cellular and intra cellular changes happening during oogenesis. In the ovary the occurrence of sex inversion has also been described. Through non denatured Native and denatured SDS-PAGE, the characterization of vitellogenin in ovarian homogenate and blood serum has been clearly elaborated. The molecular weight determination of viitellogenin protein fractions was worked out in the SDS-PAGE. To gather support data on the biochemical changes occurring during the maturation seven major parameters from four different tissues have also been studied. The information generated in this study along the above said aspects is expected to form a basis for initiating further studies on larger groupers.

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# *Introduction*

# 1. INTRODUCTION

Capture based aquaculture is receiving particular attention in maritime nations worldwide. The system, though it is an overlap between fishery and aquaculture that exploit the same resources, also has its own positive characteristics, for future development, depending on areas and species. Groupers are (*Epinephelus* spp) one group among them.

Groupers are popular food fish farmed in Southeast Asia and have potential to become an important aquaculture species, owing to their fast growth, efficient feed conversion, high market price and reduced availability from wild resources. The demand for grouper has grown markedly over the last two decades in parts of Southeast Asia ; present market value varying from 10 – 50 US$ / Kg as on 17.02.2007 in Hong Kong market (http://www.fmo.org.hk). Epinephelids of the family Serranidae constitute a very large and important group of fishes of the tropical and subtropical regions popularly known as groupers and they sustain one of the major demersal fisheries that form 2% of total marine landings in India (Paramita *et al.*, 2006). Commercially important species contributing to the grouper fishery mainly include *Epinephelus tauvina, E. bleekeri, E. chlorostigma, E. malabaricus and E. diacanthus.*

However the potential of groupers can only realized by the large scale on-shore culture activities. At present wild seed is the only source for grouper grow out system. Juveniles are available in the inshore waters and estuaries. The availability of vast inshore waters suitable for onshore farming, project the immense scope for large scale farming of groupers in India. For the semi-intensive and intensive culture practice of groupers quality seed is a prerequisite. The fluctuating and capricious nature of wild seed availability prompted investigations into captive brood stock development and hatchery seed production.

The success of any fish species is ultimately determined by the ability of its members to reproduce successfully in a fluctuating environment and thereby to maintain viable population. Reproduction is a highly integrative function, which involves complex physiological changes at intracellular and intercellular level. The reproductive gonads with the onset of maturation show a series of developmental alterations which are closely accompanied by conspicuous cellular, biochemical, molecular and endocrinological changes (Nagahama, 1983 and Guraya, 2000). These complex physiological changes control the spawning activity in natural condition. During the recent years special attention is being paid to biochemical, physiological, genetical, biotechnological and hormonal manipulation of gonadal development, sex differentiation, sex reversal and production of fertilizable gametes (i.e., eggs and sperm) as well as to development, growth and brood stock management in relation to egg and larval quality.

The majority of fish species engage in conventional reproduction, i.e., male and female exist as separate sexes (gonochorism). In addition, hermaphroditism is also quite common among fishes and a whole array of sexual patterns (accidental, rudimentary, simultaneous, protandrous and protogynous etc.) has been described in this regard (Atz, 1964; Warner, 1988; Ross, 1990). Groupers exhibit protogynous hermaphroditism in which individuals develop first as females and turn later into males with the progress of age. Information is available on biology of groupers, fattening of juveniles and sex reversal. However there is paucity of detailed and in depth information on reproductive physiology of groupers which is one of the reasons for the lag in the large scale seed production and expansion of the culture of these fishes.

Control of reproduction of candidate species is one of the most important aspects of aquaculture management. Reproductive control has multiple significance in that it helps in the quality seed production, genetic improvement of the stock and the management of natural resources. In order to proceed with the artificial means of reproduction and to produce good quality eggs, the aquaculturists have to be fully aware of the gonadal maturation stages, spawning season and the brood stock nutrition. Moreover an in depth knowledge of the biology, physiology and biochemistry of the fishes that are to be cultured is an essential prerequisite in order

to confine them in most suitable environmental conditions and to rear them with required nutritional diet. This in turn can efficiently enhance the growth of stock with the resultant increase of over all fish production. The mobilization of biochemical components due to the phenomenon like maturation, migration and spawning, which most of the fishes undergo during the life cycle have a profound effect on the body composition of fish. So, the fish body biochemistry assumed significance for healthy management of aquaculture.

Increased importance in the international market for groupers prompted many investigators to make an attempt on brood stock development of groupers. Hussain and Higuchi (1980) attempted the induced spawning of *E. tauvina* from Kuwait waters; so as *E. tauvina* in Singapore (Chen *et al.* 1977). Natural spawning and brood stock development of groupers are reported in *E. malabaricus*, *E. akara*, *E. fuscoguttatus, E. salmoides* and *E. suillus* (James *et al.*, 1997; Lim *et al.*, 1990; Toledo *et al.*, 1993; Kungvankij *et al.*, 1986 and Kohno *et al.*, 1990). Brood stock of groupers, *E. tauvina* and *E. malabaricus* were developed by rearing fingerlings under captive conditions at Cochin fisheries harbour laboratory of Central Marine Fisheries Research Institute (Mathew *et al.*, 2002; Mathew, 2005 and Anon, 1998, 1999 and 2002). Sex reversal studies were carried out in *E. malabaricus*, *E. tauvina* (Mathew, 2005) and in *E. diacanthus* (Anand, 2002). The milt of grouper *E. tauvina* was cryopreserved using a suitable cryodiluent made up of fish ringer, 5% glucose solution and cryoprotectant Dimethyl sulfoxide (CIBA Anon, 2000). Sivaram *et al*, (2004) studied growth and immune response of juvenile *E. tauvina* which were fed with herbal antibacterial active principle supplemented diets against *Vibrio harvei* infection.

Tessy (1994) studied biological aspects of *E. chlorostigma, E. bleekari* and *E. diacanthus* the three most available captive species of south west coast of India. Of these *E. diacanthus* contributed major share to the grouper catch along south west coast of India (Anon, 2000). There were reports on indiscriminate destruction and over exploitation of juvenile immature fishes of *E. diacanthus* along Dakshina kannada coast and off Quilon of Kerala (Zacharia *et al.*, 1995 and Sivakami and Seetha, 2006). *E.diacanthus* is a smaller sized species, maturing mostly around 166mm SL and captured in huge quantities in trawls and hook & lines along the

Indian coast. The maximum length reported was 502mm (Tessy, 1994 and James *et al.*, 1996). Even though many studies were carried out in *E. tauvina* and *E. malabaricus* brood stock development, little information is available on *E. diacanthus* which forms dominant share in grouper capture fisheries ( Chakraborthy,1994).

In order to proceed with the artificial means of reproduction and to produce good quality eggs it is necessary to study the gonadal maturation stages, spawning season, nutritional status of the breeders and yolk protein composition which are essential information required for the standardization of hatchery technology. It is against this background that the present study has been taken up on the reproductive physiology of female grouper, *E. diacanthus*. The information generated would form a basis for initiating further studies on larger groupers such as *E. tauvina* and *E. malabaricus*.

# *Review of Literature*

# 2. REVIEW OF LITERATURE

## 2.1 Oogenesis

Oogenesis is the process by which eggs develop and mature from the oogonia in the ovary. Oogenesis is accompanied by conspicuous cellular, biochemical, molecular and endocrinological changes (Nagahama, 1983; Guraya, 1986 and 2000). Moe (1969) utilized the criteria established by Kraft and Peters (1963), Smith (1965) and Yamamoto and Yamazaki (1961) for the description of the stages of oogenesis in red grouper, *E. morio*. Htun-Han (1978) explained the degree of ripeness of the ovary in a number of fishes.

The wall of the female gonad in general is covered externally with a peritoneal layer. The muscular tunica is composed of an intermixture of longitudinal, oblique and circular fibers. The lumen is lined with germinal epithelium which forms the surface layer of a series of longitudinal, slightly oblique folds or lamellae. They oocytes as they enlarge fill and extend the lamellae. There are no lamellae in the common oviduct or in the ventro-lateral sector of the gonad proper and as Smith (1965) suggested, this alamellar sector possibly allows the distension of the gonads with ripe eggs without injuring the germinal epithelium.

The mature ovary comprises a sac with walls consisting of ovigerous lamellae containing oocytes in the follicles and ovarian matrix. Each oocyte is delimited by an acellular envelope the chorion (vitelline membrane or zona radiata) that is surrounded by a single layer of granulosa cells and a thin thecal layer consisting of connective tissue, blood and lymph capillaries. Granulosa cells are involved in modulating the transfer of yolk precursor in to the oocyte across the chorion and in the phagocytosis of atretic oocytes. Granulosa cells and specialized theca cells produce the female sex steroids, estradiol and the maturational progesterone (Guraya, 2000).

As the oocyte begins its growth, the germinal vesicle (nucleus) presents prominent nucleoli at its periphery; these nucleoli produce large amounts of ribosomal- RNA as well as m RNAs that encode proteins required for subsequent oocyte growth such as vitellogenin receptors and yolk processing enzymes (Wallace and Selman, 1990).

Bouain and Siau (1983) described the oocyte maturation cycle showing phases of ooplasmic pH changes and of vitellogenesis in three species of groupers, *Epinephelus aeneus, E. alexandrinus,* and *E. guaza.* Yashiro *et al.* (1993) described the oogenesis and transitional changes in the gonad of grouper, *E. malabaricus.* Brule *et al.* (1999) investigated and confirmed the reproductive strategy of red grouper, *Epinephelus morio* from Southern Gulf of Mexico. Bullock and Murphy (1994) described the gonadal development of Yellow mouth grouper, *Mycteroperca microlepis.* Wyanski *et al.*, (2000) histologically examined the gonads and observed size related gonadal maturation in the snowy grouper, *Epinephelus niveatus.*

Tessy (1994) studied the gonadal development of *E. diacanthus*, *E. chlorostigma* and *E. bleekeri.* Anand (2002) observed transitional changes of gonadal germ cells with the 17α Methyl testosterone induced sex reversal in *E. diacanthus.*

## 2.2 Ultrastructural studies

Reproduction activity in most animals undergoes cyclic rhythms. The patterns of these changes in the gonads are characteristic for each species. One of the convenient methods of determining the reproductive cycle including the spawning period of a fish is to study the seasonal developmental stages in the gonads through macroscopic and microscopic observations. Macroscopic examination alone has its limitation; it may be useful for some gonochoristic fishes for gonadal stages identification but in case of hermaphroditic fishes microscopic observation is the only alternative. In macroscopic observation,

actual developmental stages of growing oocyte may not be determined. For these reasons, to understand the reproduction mechanism of any fish, a detailed histological study has become necessary.

Ultra structural studies of ovary and liver by using Transmission Electron Microscopy (TEM) has allowed a better description of cytological and nuclear processes such as yolk accumulation; formation of yolk nucleus, egg membranes, lipid droplets and cortical alveoli, while the micropyle and surface structure of the eggs has been analyzed with the help of Scanning Electron Microscopy (Flugel (1964 a, b, c); Yamamoto and Onozato(1965), Yamamoto and Oota(1967), Beeams and Kessel (1973), Riehl (1977), Brusle and Brusle(1978), Szollozi *et al.* (1978), Vanden Hark and Pute(1985), Lang (1981a,b), Ohta and Teranashi(1982), Brusle(1981, 1982, 1983, 1987 ), Wu and Sun (1983), Selmen and Wallace(1982, 1983), Kessel *et al.* (1985) and Brusle-Sicard *et al.* (1992, 1994). The functional morphology of gonads has been dealt with by Nagahama (1983), while Wallace and Selmen (1981), Wallace (1985) and Wallace *et al* (1987) have studied cellular and dynamic aspects of vitellogenesis and oocyte growth in teleosts. Upadhyay *et al.* (1978) examined ultrastructural changes in experimentally induced vitellogenesis in juvenile rainbow trout (*Salmo gairdneri* R.). Guraya (1986) gave a detailed account of cellular and molecular aspects of oocyte growth, maturation, ovulation and atresia in teleosts, while West (1990) reviewed the ultrastructural studies used for assessing ovarian development in fishes. Gopalakrishnan (1991) studied ultrastructural changes with the maturation of the gonads in female mullet, *Mugil cephalus*. Lal (1991) examined both male and female germ cell ultrastructural changes with the gonadal maturation in the protandrous fish, *Lates calcarifer*. Guraya (2000) gave a detailed description of cytological and molecular level changes with the maturation of gonads. Thiry and Poncin (2005) have studied the ultrastructural changes in the morphology of nucleolus during oogenesis in the oviparous teleost fish, *Barbus barbus*.

In understanding breeding related morpho-functional changes of the fish, liver is the first organ to be considered for study. Hepatocytes play a major role in the production of both yolk precursors and egg shell components, namely the well known vitellogenin and the zona radiata proteins (Arukwe and Goksoyr, 2003). The histomorphology of liver in teleosts differs considerably with sex and sexual activity as the organ progresses in vitellogenesis. Histological and ultra structural changes of hepatocytes will take place with the maturation of gonads (Ishii and Yamamoto (1970), Aida *et al.* (1973), Welsch and Storch (1973), Yamamoto and Egami (1974), Varghese (1976), Vander Gaag *et al* (1977), Peute *et al* (1978), Olivereau and Olivereau (1979), Van Bohemen *et al* (1981), Nunomura *et al.* (1984), Eurell and Haensly (1982), Avila (1986), Leatherland and Sonstegard (1988a, b) and Ribeiro *et al.*(2006).

In India, very few studies on ultrastructural changes in the hepatocytes with the maturation of gonads have been reported. Gopalakrishnan (1991) observed ultrastructural changes in hepatocytes with the gonadal development in female grey mullet, Mugil cephalus.

## 2.3 Hermaphroditism

The phenomenon of hermaphroditism has been observed by many workers. Dean (1923) and Herbert (1932) observed hermaphroditism in teratological condition. Lavende (1949) found protogynous hermaphroditism In *Centropristes striatus*. Spurway (1957) described first functional hermaphroditism resulting in self-fertilization in a viviparous fish *Lebistes reticulates*, and Clark (1959) reported the self-fertilisation in *Serranus subligarius*.

A fish species is hermaphroditic when a substantial proportion of the individuals among a population are able to produce gametes of both sexes, either simultaneously or sequentially, at some time during their life. Hermaphroditism may occur when the fundamental antagonism that normally exists between male and female hormonal influences during development and at maturity 'breaks down'. In this case, neither male or females sexual tissue is developmentally

preferred, or conversely switched off, and both develop (Price, 1984). For instance, in the mangrove killifish, *Rivulus marmoratus*, most young individuals contain only ovarian tissue but, with aging, there is an increasing development of testicular tissue in the gonads and so they become hermaphrodites (Cole and Noake, 1997). The potential for both male and female development exists in hermaphroditic fishes and genetic influences on hormonal control in the regulation of hermaphroditism have been discussed (Kallman, 1984; Price, 1984).

Hermaphroditism exists in three ways: (1) protogyny, in which some or all individuals function first as females and, later in life, exclusively as males; (2) protandry, in which the sex change is from male to female; and (3) simultaneous hermaphroditism. Hermaphroditism is found in at least 18 families of teleost fishes scattered through five orders ( Atz, 1964 ) and is also wide spread among the invertebrates(Ghiselin,1969). According to Atz (1964) hermaphroditism is the presence of recognizable ovarian and testicular tissue in a single individual. He described that normal hermaphroditism exists in a uniform way, at some time during ontogeny of all or many members of a species. Functional hermaphroditism is the hermaphroditism in which the individual functions both as male and female during its lifetime. In synchronous hermaphroditism the individual is capable of functioning as male and female at the same time. Here the ripe eggs and sperms develop in the gonad or gonads simultaneously. Many histological studies during decades revealed that the gonads of either protandrous or protogynous hermaphrodites have some gametes of the opposite sex; this could be confirmed when analyzing fully mature gonads (Tobin *et al.*, 1997; Zohar *et al.*, 1978, 1984; Kadmon and Yaron, 1985; Pollock, 1985; Micale *et al.*, 1987; Chang and Yueh, 1990; Chang *et al.*, 1994; Guiguen *et al.*, 1994; Micale and Perdichizzi, 1994).

The general types of hermaphroditism in the Serranidae have been shown to be a useful systematic character. Smith (1959) described synchronous hermaphroditism in four Bermuda species of the sub family Serraninae. He reported the members of the sub family Epinephelinae under protogynous on the basis of the following evidence (i) no males were found in the smaller sized

groups (ii) males appeared among medium-sized fish and the proportion increased among the higher-sized classes until the larger-sized groups were exclusively males and (iii) regressive oocytes were found in the testes of functional males.

Groupers are well known for their protogynous hermaphroditism, a condition that is derived from synchronous hermaphroditism of other serranids of the sub family Serraninae ( Smith, 1959, 1965, 1971; Tan and Tan, 1974; Chen *et al.*, 1980; Shapiro, 1987; Brusle'-Sicard *et al.,* 1992; Shapiro *et al.,* 1993; Sadovy and Colin, 1995).

Smith (1965) described three patterns of hermaphroditism in serranids; the Serranus type in which the male tissue is confined to the posteroventral region, the Epinephelus type in which the entire gonad is an admixture of ovarian and testicular tissue and the Repticus-Anthias type which is in some respects intermediate between the Serranus and Epinephelus condition. Ghiselin (1969) has stated that hermaphroditism should evolve under the following conditions, (a) where it is hard to find a mate (b) where one sex benefits from being larger or smaller than the other or (c) where there are small, genetically isolated populations.

Nakamura *et al.,* (1989) described three phases in a protogynous hermaphrodite *Thalassom druperry ;* small initial phase males (primary males), initial phase females (primary females) and large terminal phase males, which may be derived from either females which have undergone sex change to become males (secondary males). Smith (1965) described the structure of protogynous epinephelid gonad.

As in other groupers, the honeycomb grouper, *E. merra*, which have lunar synchronized spawning cycles, displays protogynous hermaphroditism and mainly inhabit the coastal waters from temperate to tropical latitudes (Lee *et al.*, 2002). In this species, the ovaries show a pattern of development similar to that described for the groups-synchronous gonochorists ( Lee *et al.*, 2002). In fact, like the rabbit fishes, which are strict lunar synchronized spawners, a mono

release of eggs may occur just before or after the major spawning lunar day ( Hoque *et al.*, 1999; Rahaman *et al.*, 2000; Lee *et al.*, 2002).

Sequential hermaphroditism (protogyny and protandry) is reported in Sapridae (Atz, 1964; Micale and Perdichizzi, 1994; Brusle-Sicard and Fourcault, 1997; Perrot *et al.*, 2000). The seabream, *Pagellus acarne*, exhibits protandric hermaphroditism, wherein all individuals first mature as males. Then, they undergo testicular regression and the initially immature ovarian zone becomes functionally female (Le-Trong and Kompowski, 1972; Lamrini, 1986; Pajuelo and Lorenzo, 1994, 2000). On the contrary, in the protogynous red porgy, *Pagrus pagrus*, males originate from females, with the ovaries showing a pattern of development similar to that described for multiple spawner gonochoristic species (males still having ovarian tissue containing some follicles capable of estrogen productions ) (Kokokiris *et al.*, 1999, 2000). Sequential hermaphroditism can be used as a population control mechanism (Moe, 1969).

Both-ways sex change (or bi-directional sex change) occurs in several polygynous species of some fish families such as Gobiidae and Pomacanthidae (Kuwamura and Nakashijma, 1998; Sakai *et al.*, 2003). Reversed sex change occurs even in fishes often demonstrating to be protogynous in nature. Indeed, it is confirmed that the larger dominant male changes sexual behavior and gonads revert back to those of a female when it becomes subordinated again, after cohabitation with a large male (Sunobe and Nakazono, 1993; Kuwamura *et al.*, 2002).

Presently, the mangrove killi fish, *Rivulus marmoratus*, is the only known self-fertilizing hermaphroditic vertebrate (Harrington, 1961; Warner, 1978; Sakkura and Noakes, 2000). This species is of interest not only for its unique reproductive biology but also because of the genetically identical individual within each self-fertilizing lineage, sometimes referred to as clones (Harrington, 1967, 1971; Harrington and Kallman, 1968). In general, mangrove killifish individuals are hermaphrodites and produce both sperm and ova simultaneously. Nevertheless, secondary males develop from the hermaphrodites by the loss of

ovarian tissue, and primary males develop directly to produce sperm throughout the rest of their lives (Harrington, 1971; Soto and Noakes, 1994).

Chacko and Krishnamurthy (1949) observed hermaphroditism in *Hilsa ilisha*. Hermaphroditism has been reported in the *Cirrhina reba* (Sathyanesan and Rangarajan, 1953), *Rastrelliger canagurta*( Prabhu and Antonyraja, 1959), *Polynemus heptadactylus*( Naik, 1960), *Katsuwonus pelamis*( Raju, 1960) and *Sardinella longiceps*(Antonyraja, 1963). Tessy (1994) identified hermaphroditism in three species of groupers, *Epinephelus chlorostigma, E. bleekeri and E. diacanthus*.

### 2.3.1 Sex reversal

Sex reversal is wide spread in a number of tropical fishes including the families Labridae, Scaridae and Serranidae. Smith (1959) has also suggested that more than one spawning season is passed in the female phase before changing sex. Liem (1963) described that in *Monopterus albus* every individual starts its reproductive cycle as a functional female and that males are produced only by sex reversal. He also found that individuals of intermediate age and length possess ovotestes. Mc Earlean and Smith (1964) suggested that the gag, *Micteroperca microlepis* mature as females during their fifth or sixth year and transform to males during their tenth or eleventh year.

Atz ( 1964 ) also described intersexuality as the presence of both male and female characteristics or of intermediate sexual characteristics in a single individual. All hermaphrodites are intersexes, but some intersexes are not hermaphrodites. Gonochorism is the existence of one sex, either male or female in the individual. Sex reversal is the change from one sex to another that is from possession of recognizable ovarian tissue to that of testicular tissue, or vice versa. Sex inversion is the acquisition by an individual belonging to one sex of characteristics similar to those of the opposite sex, but not including recognizable gonadal tissue of that sex. Sadovy and Colin (1995) mentioned the sexuality of Nassau grouper as gonochoristic with potential for sex change.

Brusle and Brusle (1975) described ovarian and testicular inter sexuality in the protogynous Mediterranian groupers *E. aenus* and *E. guaza* where the gonads were ovotestes and of Epinephelus type. They also observed that in *E. aenus* and *E. guaza* all young fishes were females and the first functional activity was oogenetic. Males occurred among the largest individuals after sex reversal. Sex reversal occurred during sexual inactivity. The period of sex reversal (ovary→ ovotestis→ testis) frequently showed transitional stages in the form of ovarian and testicular inter sexuality. Sex reversal has also been described by many workers like Warner (1975), Warner *et al.* (1975), Thompson and Munro (1978), Bruce (1980), Hastings (1981), Young and Martin (1982), Cole (1983), Abu Hakima (1984), Hasting and Peterson (1986), Mackie (2006) etc.

Aldenhoven (1986) observed that females of protogynous, *Centropyge bicolor* may be induced to change sex by recruitment of a threshold number of additional adult females into the group even in the presence of the male. The presence of yellow-brown bodies in the testes was used uncritically in the past to conclude in favor of hermaphroditism (Sadovy and Shapiro 1987). Shapiro (1981, 1987) found that social environment has a control over the sex in the case of coral reef fishes.

Fisher and Patersen (1987) gave an account on the evolution of sexual patterns in the seabasses. Cole and Robertson (1988) have not observed any remnants of ovarian tissue in *Coryphopterus personatus*, a protogynous hermaphrodite. The sperm produced are transported through a newly formed, permanent system of anastomosing tubules that comprise the body of the gonad.

In the gold lined seabream, *Rhabdosagrus sabra*, a protandrous hermaphrodite, the plasma pattern of sex steroids is similar in male and inter sex individual. However, the intersex individuals have higher levels of conjugated estradiol, of conjugated androstenedione, and of free testosterone, than do the females, even during the spawning period. This may indicate that to have a sex change from male to female, the estrogen levels have to be higher to suppress

the antagonistic effects of androgens (Yeung *et al.*, 1993). Nevertheless, some species display the normal teleost pattern of steroid levels, with higher levels of serum 11-keto testosterone in males than in females (Nakamura *et al.*, 1989, 1994; Cardwell and Liely, 1991a, b; Cochran and Grier, 1991; Godwin and Thomas, 1993).

In most serranids sex inversion occurs at an advanced age, rendering males difficult to capture from the wild and handle in captivity. Sex reversal has been described for several species of groupers belonging to the genus *Epinephelus*: for example, *E. tauvina* (Chen *et al.*, 1977) *E. fario* (Kuo *et al.* 1988), *E. bruneus*, *E. septemfasciatus* and *E. akaara* (Lee, 1995). Several studies have demonstrated the use of androgens to induce sex change in captive groupers (Roberts and Schlieder, 1983; Kuo *et al.*, 1988; Tan – Fermin, 1992; Hassin *et al.*, 1997; Tan-Fermin *et al.*,1994; Quinitio *et al.*, 1997, 2001). Among the administration methodologies used, androgen implantation seems to be the most effective with respect to the success of sex change, cost of hormone and survival (Yeh *et al.*, 1988, Yeh and Ting, 1990; Chao and Lim,1991; Yeh *et al.*, 2003). Natural sex reversal observations in hermaphroditic fishes are very few. Lone and Al-Marzouk (2000) observed natural sex reversal in protandrous Bream ( *Sparidentex hasta*) from Kuwait waters.

Mathew *et al.* (2002) reported the induced sex reversal in *E. tauvina* and *E.malabaricus,* by oral administration of 17α-Methyl testosterone. Anand (2002) studied induced sex reversal in *E. diacanthus* by 17α-Methyl testosterone containing pellet feed administration. He could see the transformation of females to males within three months of androgen induction (personal communication).

## 2.4 Vitellogenesis

Vitellogenin is the major yolk precursor protein in non mammalian vertebrates and invertebrates (Deeley *et al.*, 1975; Gap *et al.*, 1979; Korsgaard and Petersen, 1979). In vertebrates, it is produced in the liver in response to estrogen stimulation (Ansari *et al.*, 1971; Plack and Fraser, 1971; Sundarraj and

Nath, 1981). Vitellogenin secreted by the liver is transported by the blood to growing oocyte, where it is sequestered and protelytically cleaved in to the yolk proteins, mainly lipovitellin and phosvitin, during the period of gonadal development (Selman and Wallace, 1982).Ultra structural studies of vitellogenic follicles of fishes injected with horseradish peroxidase have demonstrated a possible route for the vitellogenin passage from the blood capillaries via the extra cellular space among theca cells , across the basal lamina, through the spaces between adjacent granulosa cells , around the microvillar projections of the granulose and those of the oocyte , and across the canals in the chorion to make contact with the oolemma (Wallace and Selman, 1990). The main bulk of the fully grown oocyte is due to yolk accumulation from its precursor's vitellogenin. Lipovitellin is the major yolk protein product and contains about 20% lipids by weight (Matsubara *et al.*, 2003).

Yolk proteins are stored in the oocyte of teleosts in the form of globules or granules (Folmar *et al.*, 1995). Yolk proteins are rich in poly unsaturated fatty acids. They primarily serve as metabolic energy reserves. It would appear that vitellogenin transports mainly structural lipids and essential fatty acids into the oocytes that support embryo tissue growth, where as neutral lipids derived from the hydrolysis of other circulating lipoproteins, are utilized as energy reserves for embryonic and larval life (Patino and Sullivan, 2002).

### 2.4.1 Vitellogenin isolation and Characterisation

Vitellogenin has been isolated from the blood of a number of teleost fishes (Ng and Idler, 1983). A number of different techniques have been established to enable blood vitellogenin levels to be determined. De vlaming *et al.* (1980) used a chromatographic procedure for isolating intact, highly purified vitellogenin from the plasma of the gold fish, *Carassius auratus*. Craik and Harvey (1984b) developed an alternative alkali-labile protein linked phosphorus measurement method for indirect estimation of yolk protein. Copeland *et al.* (1986) found that immunoassayable vitellogenin in plasma from male rainbow trout had the same molecular weight as authentic vitellogenin from female fish. He also stated that

vitellogenin levels of pre-spawning female trout was two hundred fold higher than males of the same strain and age. Black and Skinner (1987) studied the plasma lipoprotein level changes with the spawning in rainbow trout.

In a number of piscine species female specific serum lipoproteins (vitellogenin) have been detected by electrophoretic, chromatographic, gel fitration or ultracentrifugal procedures during the vitellogenic period of the annual reproductive cycle (Hickey and Wallace, 1974; Hara, 1975; Emmersen and Petersen, 1976; de Vlaming *et al.*, 1977; Masurekar and Pai, 1979; Gopalakrishnan, 1991; Lal, 1991; Utarabhand and Bunlipatanon, 1996; Heppell and Sullivan, 1999 and 2000; Heppell *et al.*, 1999). It has been observed that antiserum developed against oocyte or egg extracts from various piscine species cross reacts with serum of vitellogenic females but not with serum of males or immature females ( Emmersen and Emmersen, 1976; de Vlaming *et al.*, 1977; Emmersen *et al.*, 1979; Le Menn, 1979). Gopalakrishnan (1991) observed similar banding pattern of ovarian homogenate with vitellogenic female serum in the grey mullet, *Mugil cephalus*.

Vitellogenin is a high molecular weight protein ranging from 250KD to 600KD in different species. It contains variable amount of lipids (Norberg and Haux, 1985), carbohydrates and phosphate, thus it is defined as a phospholipoglycoprotein. Vitellogenin has been characterized in several species (Hara and Hirai, 1978; Campbell and Idler, 1980; de Vlaming *et al.*, 1980; Nath and Sundar raj, 1981; Chan *et al.*, 1991; Gopalakrishnan, 1991; Heppell and Sullivan, 1999). Utarabhand and Bunlipatanon (1996) isolated and identified the plasma vitellogenin from the grouper, *Epinephelus malabaricus*. The vitellogenins of both cat fish (Bradley and Grizzle, 1989) and tilapia (Chan *et al.*, 1991) have been tentatively identified as being a single polypeptide, where as those of gold fish (de Vlaming *et al.*, 1980) and Japanese eel (Hara *et al.*, 1980) appear to be composed of three or four subunits.

## 2. 5 Biochemical composition

Biochemical composition of fishes which is an indicator of the nutritive value is subject to variations depending on seasons, food intake, breeding and migration. Attainment of gonadal maturity marks a change in the growth pattern of fish, resulting from the "reproductive drain "of materials to somatic growth of the gonads. Proximate composition analysis of fish during the gonadal development can also disclose energy sites of various nutrients in the body and trace out the pathway through which they are mobilized to the gonad. From aquaculture point of view, a thorough understanding of the biochemical composition of the parent fish keeps us informed about their nutritional requirements for their captive breeding to develop the aquaculture industry of candidate species. Egg viability and larval survival can also be determined by the biochemical composition of the egg (Srivastava and Brown, 1991; Mackenzie *et al.*, 1996; Pickova *et al.*, 1997; Riveiro *et al.*, 2000).

Studies on variations of biochemical composition of different tissues with the maturity of gonads will give information on brood stock nutrition needed for management of capture fisheries of candidate species. Investigations on the biochemical constituent changes of different tissues of teleosts during pre-spawning, and post spawning periods have been observed with special reference to moisture, protein, lipid, carbohydrates, cholesterol, ash and carotenoids(Robards *et al.*, 1999; Craig *et al.*, 2000; Hernandez *et al.*, 2003). Krinsky (1994) reviewed the biological properties of carotenoids and felt the need of further research to make a firm case on the positive effect of dietary or supplemental carotenoids on improving fertility or reproductive capacity in a number of animals.

Proteins are large, complex molecules made up of various amino acids which are essential components in the structure and function of all living organisms (NRC, 1983). Egg Viability and Larval survival can also be determined by the biochemical composition of the egg. Larval survival increased as the egg protein percentage increased (Srivasthava and Brown, 1991; Mackenzie *et al.*,

1996; Pickova *et al.*, 1997; Riveiro *et al.*, 2000). Many investigators have found out a close relation to protein content variations in different tissues of the fish with the maturation of the gonads (Sivakami *et al.*, 1986; Muthukaruppan, 1987; Gopalakrishnan, 1991; Lal, 1991; Robards *et al.*, 1999).

Lipids have been recognized as a major form of energy storage in the muscle, liver, intestine and gonads. In the reproductive season, the fish depend on these energy reserves for the growth and development of the gonads (Damberge, 1964; Gras *et al.*, 1967). Comparative study of seasonal lipid changes in two populations of Brook Char, *Salvilinus fontinalis* showed that there is association between lipid changes and reproductive out put (Nelson and McPherson, 1987). Oocyte acquisition of lipids from source other than vitollogenin dominates follicle growth in many teleosts (Patino and Sullivan, 2002). El-Sayed *et al.*, (1984) investigated the seasonal variations of total lipids, free fatty acids, triglycerides, phospholipids and cholesterol content of the fresh water fish *Tilapia nilotica* and marine fish *Sparus auratus* . Energetically, lipids, were the predominant source of energy for embryonic development (Mac Farlano and Nolton, 1999). Lipids of fishes are subjected to seasonal variations reflecting the physiological status of the animal. Fish accumulate lipid reserves to be used in part, either as an energy source during the winter season or for gametogenesis and gonadal maturation (Chartzifotis *et al.*, 2004).

Cholesterol is an important precursor of steroid hormones and is a constituent of cell walls. Serum cholesterol levels are minimal at the time of the greatest sexual conversion to sex hormones. Shreni and Jaffari (1974) have suggested that the egg cholesterol reflects the parental food in take. Sen and Battacharya (1981) reported the cholesterol level changes of the gonads in relation to maturation. Farrell and Muni (1983) observed that the cholesterol levels of cage reared *Salmo salar* was high compared to sea ranched individuals especially during the spawning run. El-Sayed *et al.* (1984) investigated the seasonal variations of total lipids, free fatty acids, triglycerides, phospholipids and cholesterol content of the fresh water fish *Tilaia nilotica* and marine fish *Sparus auratus*. Diwan and Krishnan (1986) observed the serum cholesterol level changes with the gonadal development in both male and female *Etroplus*

*suratensis*. Muthukaruppan(1987) mentioned the cholesterol changes with the maturation of gonads in *Liza parsia*. Gopalakrishnan(1991) studied the cholesterol content changes in body tissues and blood tissues of the female grey mullet, *Mugil cephalus*. Lal (1991) examined the cholesterol level changes with the maturity stages of both male and female gonadal tissues in the protandrous hermaphrodite, *Lates calcarifer*.

Carbohydrates act as a cheap energy source that would spare catabolism of other components such as protein and lipids to energy ( Sales and Janssens, 2003). Though glycogen levels in the muscle, liver and gonad are comparatively low, its role in mobilization in energy during maturation and spawning may not be considered negligible. Mobilisation of glycogen in the ovary has been reported by several workers ( Venugopalan, 1962; Sivakami *et al.*, 1986; Muthukaruppan , 1987; Gopalakrishnan, 1991 ).

The liver is the site of synthesis of lipoproteins ( Leger, 1985) and it is a major storage organ for Vitamin-A (Al-khalifa and Simpson, 1988). Hardy *et al.*, (1990) observed that the salmonoid fish liver, including that of charr is the major organ for transferring carotenoids to the reductive metabolites. It has also been established that ingested carotenoids accumulate in the liver and flesh and are mobilized to the gonads as they ripen. Crozier (1970) examined the tissue carotenoids in pre-spawning and spawning sockeye salmon (*Oncorhynchus nerka*). The redistribution of carotenoid from the liver and flesh to gonad has also been noticed in *Salmo trutta, Perca fluviatillis*, *Luciscus cephalus*, and *Barbus barbus* ( Shnarevich and Sakhnenko, 1971). The relative increase in size of the liver along with gonad maturation has been found by Krivobok (1964). In Baltic cod (*Gadus callarias*), females with large livers or liver rich in lipid, spawn eggs containing more lipid than usual (Grauman, 1972).

The role of carotenoids so often found in the ovarian tissue has been much discussed. Duefel (1975) has shown that carotenoids fulfill an important function in reproduction. These terpene compounds are deposited in the ovary during maturation and are distributed with the function of imparting coloration and viability to the eggs. Accumulation of carotenoids in the gonads during the

process of maturation has been observed by Shnarevich and Sakhnenko(1971). Matsuno *et al.,* (1985) have reported on the changes in composition of carotenoids during maturation of with marine eggs. Torrissen and Torrissen (1981) have observed high mortality with the lowest carotenoid content for the eggs of Atlantic salmon. Numerous workers have reported on the relation between the degree of egg pigment and mortality during the embryonic development ( Hirao *et al.*, 1954 a, b; 1955 a, b; Borovik, 1962; Galkina, 1969; Gergiev, 1971; Torrissen and Torrissen, 1981; Tacon, 1981) . According to Springate *et al,* (1984), the percentage of successful fertilization is sometimes used as the sole measure of egg quality since; it is highly correlated with both hatching and first feeding success. Patnaik (2001) studied the carotenoids mobilization with the maturation of gonads in *Priacanthus hamrur.*

Minerals are inorganic elements required by fish for tissue formation and various functions in metabolism reproduction and regulations (Sales and Janssens, 2003). Studies reported on seasonal changes of mineral content with the maturation of gonads are many (Muthukaruppan, 1987; Gopalakrishnan, 1991; Lal, 1991; Robards *et al.*, 1999; Hernandez *et al.*, 2003).

Biochemical composition of fishes is influenced by several factors including patterns of feeding, gametogenesis and probably environmental conditions. Mobilization of macronutrients takes place during the spawning period from the fish muscles and liver to the developing gonads (El- Sayed *et al.*, 1984). He also examined the seasonal variations of total lipids, free fatty acids, triglycerides, phospholipids and cholesterol contents of the fresh water fish *Tilapia nilotica* and marine fish Sparus *auratus.*

Several studies have been carried out on the correlation of biochemical constituent mobilizations with the gonadal maturation (Plack and Woodhead, 1966; Fletcher and King,1978; Masurekar and Pai, 1979, Craik and Harvey, 1984b and 1987; Jayashree and Srinivasachar,1979; Sheridan *et al.*, 1983; Kozlova, 1997; Chowdhury and Joy, 2000)

The above review indicates that most of the works carried out are on sex reversal and reproductive biological aspects of the groupers. To get proper control over captive breeding of groupers we must know what is the physiological condition of the fishes when the fish gonads under go maturation. Generation of information over these aspects is very much essential for standardization of breeding technology of groupers. The present study was planned to answer some of the questions especially in a cultivable commercially important fish group- 'the groupers'.

# *Material and Methods*

# 3. MATERIAL AND METHODS

The present study was carried out at Central Marine Fisheries Research Institute, Cochin from September 2002 to August 2004.

### 3.1 The selected species

The study was carried out in the species ***Epinephelus diacanthus*** (Valenciennes, 1828) (Plate. 1). The fish belongs to the order perciformes; family Serranidae in the sub family Epinephelinae. The fish has distinctive characters of dorsal fin with 11 spines and 15-17 soft rays; anal fin with 3 spines and 8-9 soft rays; pectoral fin rays 18-20; caudal fin rounded to almost truncate, The body depth 2.8-3.2 times in standard length. Preopercle border forming nearly a right angle with 1-3 enlarged serrae at the angle; sides of lower jaw with 2 rows of small sub equal teeth; anterior nostrils tubular, with large flap posteriorly extending over the rear nostril.

**Colour**

Body pale grayish brown, usually with 5 dark vertical bars; 4 bars below dorsal fin and 5$^{th}$ on caudal peduncle. Ventral part of head and body often pink or reddish in colour. The fish occurs in mud or muddy sand bottom in depths of 10-120 m. The species attains 52 cm in total length ( Fisher and Bianchi,1984; Heemstra and Randall, 1993).

Common name: **Spiny cheek grouper or Thorny cheek grouper**
Vernacular name: **"Kalava".** (Malayalam)

### 3.2. Collection and preservation of sample

#### 3.2.1. Collection sites:

Live specimens of *Epinephelus diacanthus* were collected onboard Fishery Survey of India vessel (Plate. 2) during the cruises off Quilon region (latitude: 8° 55'N and longitude: 76° 30' E) (Fig. 1) and off Ratnagiri region (Latitude: 15° 42' N and Longitude: 73° 16' E) at the depth of 50 meters. Fishes were also collected from local trawlers operated off Quilon region.


PLATE 1. *Epinephelus diacanthus* (Valenciennes, 1828)

Plate 2. FSI vessel

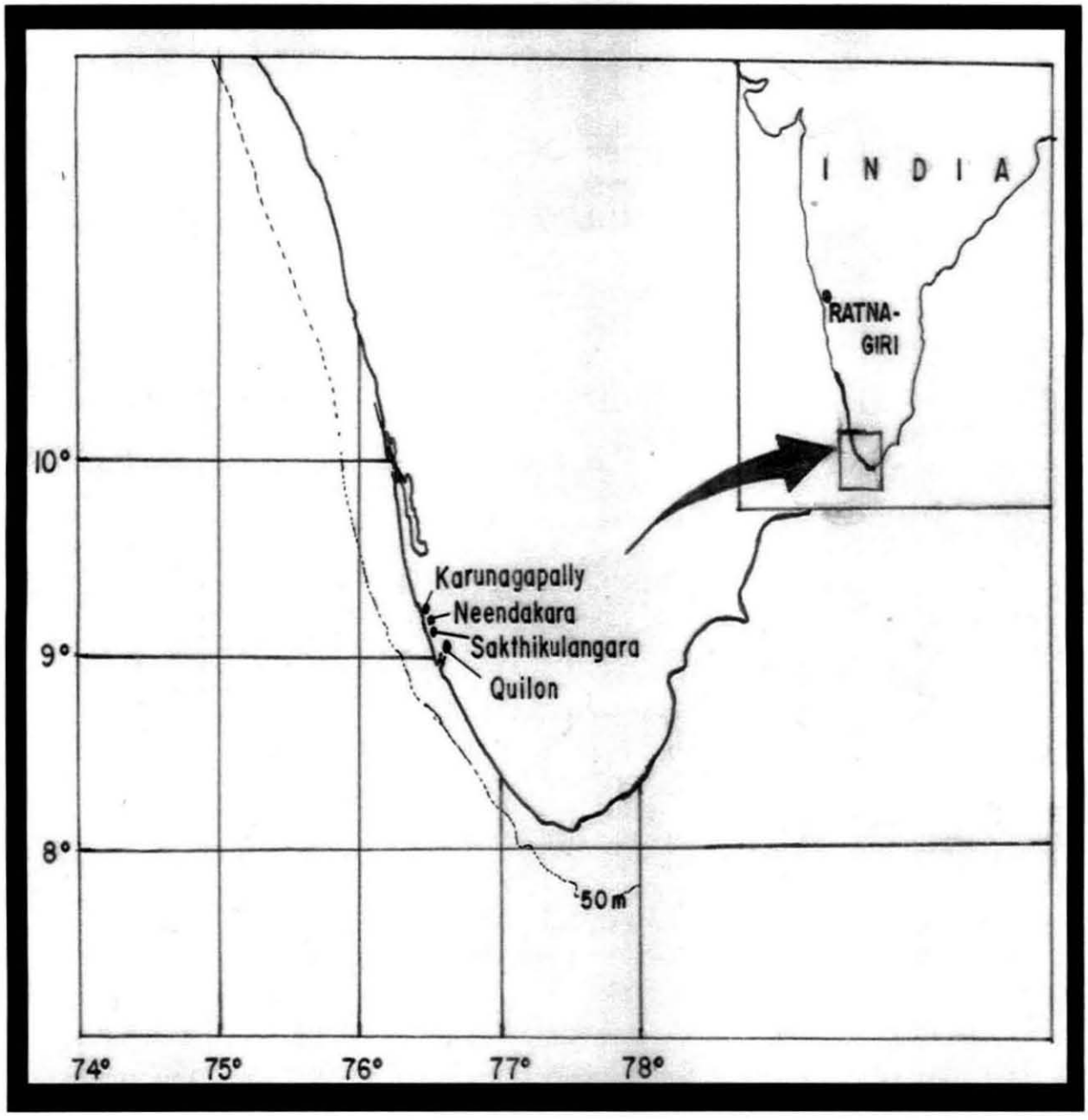



Fig. 1 Specimen collection sites

### 3.2.2. Collection methods and preservation:

Live fishes were collected onboard Fishery Survey of India vessels and were transferred to buckets containing sea water mixed with 100ppm of anesthetic clove oil (Koya, 2003). The fishes were measured accurately to nearest millimeter (mm) for total length, standard length and total weight. Each fish was dissected to remove the liver, gonads and muscle tissue from below the first dorsal spine (care was taken not to include the skeletal parts in the muscle sample). The dissected tissues were covered with aluminum foil , labeled and packed in labeled 4X5cm polythene bags. The polythene bags were preserved at -20° C until the landing of vessel. After reaching the shore all the samples were loaded in the ice box and transferred to the laboratory. Tissues were immediately placed in deep freezer. The liver and ovary preserved in polythene bags were taken and their weights were recorded up to milligram level with an electronic balance (Sartorius balance) for the determination of Gonado Somatic Index (GSI) and Hepato Somatic Index (HSI) of each fish. Then the polythene bags were placed back in deep freezer until further studies were carried out.

After complete anesthetization of fishes, individual fishes were taken for blood collection. Disposable syringes with heparinised needles, of 5ml quantity were used for the blood collection. Blood was collected by a cardiac puncture. Immediately after collection of blood the needle was removed and blood expelled into clean glass tubes and allowed for settlement of the blood cells in atmospheric conditions. After this the clear solution separated from the blood was transferred to clean labeled eppendorf tubes. Then the eppendorf tubes were kept in deep freezer (-20° C temperature) for preservation.

The collected clear solution from the blood was centrifuged at 3000 rpm for 20 minutes. Then the supernatant straw coloured serum after centrifugation was transferred to clean dry eppendorf tubes. Samples wherein haemolysis was noticed were discarded. Serum samples were kept preserved at -30° C until further studies were carried out.

Ovaries of fishes collected onboard were carefully observed in the laboratory under stereoscopic dissection microscope to study their maturity stages.

### 3.3. Biological parameters

The fishes were cleaned and the excess water on the body was blotted out. They were measured up to the nearest millmeter (mm) for their total length. The specimens were then cut open and the gonads examined. The gonads were assigned to three different maturity stages as suggested by Qasim (1973).

#### 3.3.1. Oocyte size – frequency profiles

Oocyte diameter measurements were taken from ovaries belonging to various developmental stages and oocyte size-frequency profiles were constructed, with a view to trace the development of ova from immature stage to ripe condition (Clark, 1934; Prabhu, 1956; Greeley *et al.*, 1987). A representative piece from the collected ovary was gently teased on a clean dry glass slide and spread over it. The diameter of the egg was measured along the horizontal axis using an ocular micrometer, which was calibrated using a stage micrometer (Each ocular division was found to be equal to 0.025 mm). The measurements were classified into 50 /um. The ova were pooled from anterior, middle and posterior portions of the ovary (Tessy, 1994).

#### 3.3.2. Gonado-somatic Index (GSI):

The Gonado-somatic Index (GSI) for each fish was calculated using the formula of June (1953) and Yuen (1955).

$$GSI = \frac{\text{Wet weight of the ovaries}}{\text{Total body weight of fish}} \times 100$$

The range and average values of GSI were determined for each maturity stage.

#### 3.3.3. Hepato-somatic Index:

The Hepato-somatic index (HSI) was calculated using the formula suggested by Crupkin *et al.* (1988).

$$HSI = \frac{\text{Wet weight of whole liver in grams}}{\text{Total weight of fish in grams}} \times 100$$

The range and average values of Hepato Somatic Index at each maturity stage were calculated.

### 3.3.4. Condition Factor 'K':

The' ponderal index 'or 'condition Factor,' K' for each fish was calculated using the formula suggested by Clark (1934).

$$K = \frac{W}{L^3} \times 100$$

Where, W = Total weight of fish in grams

L = Total length of fish in cm.

The range and average values of 'K' were determined for each maturity stage.

### 3.3.5. Fecundity

The potential fecundity or the number of eggs available for spawning in a single breeding season, was estimated from the ripe ovaries. These estimates were based on sub sampling of unbiased samples of ovaries from gravid fish collected during the peak spawning period as recommended by Begenal and Braum (1978). Sub samples weighing about 200mg were taken from the anterior, middle and posterior regions of the bilobed pre-weighed ripe ovary and placed on a glass slide with a drop of 1% Formalin in 0.6% Nacl. The individual eggs were separated and the number of yoked eggs was counted under a dissection microscope. The fecundity of the fish was determined using the formula:

$$F = nG / g$$

Where F = Fecundity
n = number of eggs in the sub sample
G = Total weight of paired ovaries in grams and
g = weight of the sub sample in grams.

The relation ship between the fecundity (F) and total length (L); fecundity and total body weight (W), and fecundity and total gonad weight of the fish were determined using regression equations.

## 3.4. Histological studies

The process of oogenesis was studied by utilizing histological preparation of ovaries from females belonging to different gonadal maturity stages with haematoxylin and eosin staining.

### 3.4.1. Sample Fixation

After dissecting the ovary from fresh fish ovary sample was cut into pieces for easy penetration of fixative. The ovary pieces from fresh specimen were fixed in Bouin's fixative for 24 hrs. The tissues were then washed under running tap water for 8 - 10hrs and stored in 70% ethyl alcohol until further processing.

### 3.4.2. Processing and sectioning

The 70% ethyl alcohol preserved tissues were transferred to the above graded alcohol series i.e., 80%, 90% and 100 % (Weesner, 1960), and kept in xylene until they became transparent. They were then transferred to xylene II followed by xylene-wax mixture. Then the tissues were impregnated with and embedded in molten wax (58°C melting point). Tissue blocks were prepared and serial sections were cut at 5μ in a rotary Leica microtome. Serial sections were transferred to waterbath to spread out uniformly without foldings. Then the sections were spread over glass slides on which the adhesive Mayer's albumen was applied (Gray, 1973). They were later deparaffinised in xylene, hydrated, stained with Delafield's heamatoxylin, counter stained with 1% aqueous eosin, dehydrated and mounted in DPX.

### 3.4.3. Photomicrography

The permanent slides prepared were photographed with the help of camera attached to a Leica binocular microscope (leica, LB-50).

### 3.5. Ultra structural studies

Ultrastructural studies were carried out in Hitachi-H-600 Transmission Electron Microscope (Hitachi Ltd, Tokyo, Japan). The tissue preparation and processing was done as per Dawes (1988). Ovary and liver were analyzed for ultra structural changes with maturation of the fish.

#### 3.5.1. Fixation

Tissue cut into one mm cubes from ovary and liver at different stages of maturation of the fish were excised from anaesthetized fishes and immediately transferred to chilled 3% gluteraldehyde solution in 0.1M cacodylate buffer. After 12hrs, tissues were removed from the fixative and allowed for washing and then transferred to cacodylate buffer, pH 7.3. The tissues were kept in buffer at 4°C until the vessel reached land. After the vessel landing the fixed tissues were washed in 0.1M sodium cacodylate buffer three times (30min each) and kept overnight.
Tissues were post fixed in 1% osmium tetroxide (in cacodylate buffer) for 2hrs at 4°C. The tissues were again washed three times (30min each) in buffer.

#### 3.5.2. Dehydration and Embedding

Dehydration of tissues was carried out in ascending grades of acetone (Annexure.1) at 4°C and embedded in Spurr's resin as per the method described by Spurr (1969).

#### 3.5.3. Sectioning and staining

Ultra thin sections (60 – 90nm) were taken in a LKB Nova Ultra microtome (LKB – producter AB, Sweden) using glass knife. These sections were lifted on to matted surface of copper grids (300-mesh size). The sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshally, 1965), dried and observed under transmission electron microscope.

### 3.6. Electrophoresis

For separation and characterization of vitellogenin from crude ovarian homogenate and serum proteins, vertical native PAGE (Davis, 1964) was employed.

#### 3.6.1. Standardisation of PAGE

Discontinuous electrophoresis was carried out with a stacking gel of 4 - 8%; resolving gels of 6 - 12% were tried to choose an ideal percentage. Finally 10 % concentration gave a better separation and this concentration was selected for further studies (Table-1&2).

#### 3.6.2. Casting of Gel

The resolving gel components were mixed gently and poured in to the prepared cassette. Few drops of butanol were over layered to prevent meniscus formation and gel was left undisturbed to set. After polymerization of the resolving gel, the overlying butanol was removed and the cassette was washed with double distilled water and dried. The prepared stacking gel mixture was then poured over the separating gel. The comb was placed in the stacking gel and allowed to set. After the gel got polymerized, the comb was removed without distorting the shape of the well.

#### 3.6.3. Ovarian homogenate sample preparation:

Ovarian homogenate proteins were extracted, following the method of Hara *et al.* (1980) with little modification. The appropriate tissue weight was determined after standardization and 200mg ovary /ml of solvent gave good resolution of bands. Tris buffer ($p^H$-8.3), Ice cold double distilled water and 100mM Phosphate buffer ($p^H$-7.2) were tried as solvents. Phosphate buffer gave good resolution and it has taken as solvent for further studies. During the extraction of protein from ova, the outer ovarian membrane was removed and the weighed sample was washed in double distilled water. It was then thoroughly homogenized in ice cold 100mM phosphate buffer ($p^H$-7.2) containing 0.01% Phenyl methyl sulfonyl fluoride (PMSF) as a protease inhibitor using a mechanical homogenizer. The contents were centrifuged at 10000rpm for 30 minutes at 4°C in a refrigerated centrifuge. The supernatant was

Table-1
Reagents composition used for gel running

| | PAGE | | |
|---|---|---|---|
| 1. | Stock acrylamide solution<br>Acrylamide<br>Bisacrylamide | <br>29.10g<br>0.90g | <br>100ml double distilled water |
| 2. | Resolving gel buffer<br>(pH 8.8)<br>1.8M Trisbase | <br><br>18.2g | <br><br>100ml double distilled water |
| 3. | Stacking gel buffer<br>( pH 6.8) | <br>6.1g | <br>100ml double distilled water |
| 4. | 10% Ammonium persulphate prepared freshly every time of gel run | | |
| 5. | TEMED | | |

Table – 2
Composition of resolving and stacking gel used in the Native PAGE

| 10% Resolving Gel | 6.5% Stacking Gel |
|---|---|
| Acrylamide stock solution - 13.3ml | Acrylamide stock solution - 2.7ml |
| Resolving gel buffer (pH – 8.8) – 10.0ml | Stacking gel buffer (pH – 6.8) – 3.125ml |
| Ammonium per sulphate - 400µl | Ammonium per sulphate - 125µl |
| TEMED - 28 µl | TEMED - 8.75 µl |
| DDW - 16.24ml | DDW – 6.535ml |

collected and filtered through a filter paper (Whatman No.41). This filtrate was again centrifuged at 10000rpm for 30 minutes at 4°C and the clear supernatant was collected and stored at -30°C until further analysis. Male gonadal tissue (mature stage) were also processed in the similar manner for protein extraction.

### 3.6.4. Sample application and Electrophoresis

Sample was prepared by mixing 20 µl of ovarian supernatant with 20 µl of loading buffer. The gel was carefully removed from the gel casting unit and set on the electrophoretic apparatus, with the notched plate facing the upper tank, using screws. Electrode buffer was poured in the tanks. Care was taken to avoid entrapment of air bubbles at the bottom of the gel. The prepared samples were applied to the wells in the stacking gel and were layered with running buffer in order to avoid disturbance of the sample. The electrodes were then connected to the power pack. Electrophoresis was conducted at a constant voltage of 50V; until the dye front crossed the stacking gel then was increased to 140V. The entire run was carried out at 4°C till the dye front reached the bottom of the gel.

### 3.6.5. Staining the gels

Native PAGE gels were removed from the plates and subjected to an overnight fixation. This was followed by staining, for 2hrs in Commassie Brilliant Blue R-250, to study the general protein profile. The excess stain was washed off and the gels were finally immersed in destainer (Table-3). For characterization of lipoprotein band, the gel was stained for carbohydrate, lipid and calcium moieties, with periodic acid Schiff's reagent, sudan black B and Alizarin red's', respectively, as shown in Table-3.

Table - 3
Details of staining techniques adopted in electrophoresis

| TESTS | FIXATION TIME | STAINING TIME | DESTAINING SOLUTION AND DURATION | COLOUR OF BAND |
|---|---|---|---|---|
| **1. PROTEIN**<br><br>Commossie Brilliaxt blue<br>(Davis, 1964) | 10% TCA 30 minutes | 30 minute in dark | Methanol, water of acetic acid (5:5:1) for 30 minutes | BLUE |
| **2.POLYSACCHARIDAE**<br><br>Periodic acid Schiff's reagent<br>(Gordon, 1980) | 12.5% of TCA for 30 minutes | 1 hour in 1% periodic acid in 3% acetic acid; 1 hour wash in DDW; 3 hour in Schiff's reagent in dark at 4 C | 1% aqueous Sodium metabisulphite followed by repeated washing in 7% acetic acid | Magenta |
| **3. LIPOPROTEIN**<br><br>Sudan Black B<br>Prat *et al.* (1969) | | 2 hours | Ethanol for 30 min. | Brown Black |
| **4. CALCIUM**<br><br>Alizarin's red<br>(Dahl, 1952) | Ethanol (1-2h) | 20 minutes in Alizarin's red staining solution adjust (pH-6.5) | Repeated washing in DDW; rinse in the acid ethanol (10 M Hcl in 95% ethanol) until background is clear | Deep reddish orange |

### 3.6.6 SDS PAGE Analysis of serum yolk protein

Serum yolk proteins of mature / ripe female fish were analyzed by SDS PAGE on 12% resolving gel and 6% stacking gel as described by Laemmli (1970) using a Midi vertical slab gel electrophoresis unit (Bangalore, GeNei) with gel dimensions of 16cm X14cm and a gel comb with 13 wells.

Electrophoretic separation of proteins was carried out using 12%(W/V) acrylamide resolving gel (29.2% acryl amide, 0.8% Bis acryl amide and 0.1% SDS) and 6% stacking gel (30% acryl amide, Bis acryl amide 0.8% and 0.1% SDS) (Table – 4). The serum sample of 20μl was mixed with five times Laemmli sample buffer in a proportion of 1:5 and subsequently heated at 95°C for 5 minutes for sample and 2minutes incase of molecular marker before loading into wells and electrophoresed at a constant voltage of 50V; until the dye front crossed the stacking gel; then it was increased to 140V and continued until the dye front reached the bottom of the gel. Standard medium range molecular marker (GeNei, Bangalore) was run parallel along with serum proteins to determine the relative molecular weight of the polypeptides. After electrophoretic run the protein bands were visualized by staining with Coomassie brilliant blue- R250.

### 3.6.7. Determination of molecular weight

The gel documentation, molecular weight estimation of respective protein bands were carried out with Biorad software enabled gel documentation unit. Rf values (Relative fraction) of respective protein bands in the native gel was also worked out with it.

Table – 4

Composition of resolving and stacking gel used in the SDS-PAGE

| 12% Resolving Gel | **6% Stacking Gel** |
|---|---|
| Acrylamide and bis acrylamide- 12.0ml | Acrylamide and bis acrylamide - 2.5ml |
| Resolving gel buffer (pH – 8.8) – 6.0ml | Stacking gel buffer (pH – 6.8) - 3.125ml |
| 10%SDS - 300 µl | 10%SDS - 125 µl |
| Ammonium per sulphate - 400µl | Ammonium per sulphate - 125µl |
| TEMED – 30.0 µl | TEMED - 8.75 µl |
| DDW - 11.57ml | DDW – 6.61ml |

## 3.7. Biochemical Analysis

Moisture, total protein, total carbohydrates, total lipids, total cholesterol, total carotenoids and ash contents in the muscle, liver and gonad tissues of *Epinephelus diacanthus* were estimated during different stages of maturation. The serum was also analaysed for all the above biochemical parameters except for moisture and ash contents.

### 3.7.1. Estimation of moisture content

The moisture content of ovary, liver and muscle tissues was determined by keeping pre-weighed wet samples at 70°C in hot air oven till constant weights were obtained. The loss in weight was taken as the moisture content and expressed as percentage.

### 3.7.2. Estimation of total protein

To estimate total protein content of the tissues, Lowry *et al.* (1951) method was followed. Pre-weighed 25mg wet tissue samples of muscle, liver & ovary sample were homogenized with 1 ml of deproteinising agent (10 % trichloroacetic acid) by keeping the tubes in ice. In the case of blood serum samples, 0.1 ml of serum was mixed well with 1 ml of deproteinising agent. Then the solution kept for centrifugation for 20 minutes at 3000rpm. The centrifugation was carried out at 4°C. Supernatant was used for estimation of carbohydrates. The protein precipitate in each tube was dissolved in 5 ml of 1N NaOH. From the above solution 1ml was taken and added to 5 ml alkaline mixture freshly prepared (50 ml of 2% $Na_2CO_3$ in 0.1 N NaOH and 1ml of 0.5 % $CuSO_4$. 5 $H_2O$ in 1 % Sodium tartrate). Then the solution was kept at room temperature for 10 minutes.

After 10 minutes 0.5 ml of IN Folin-Ciocalteau's reagent was added to the above solution (diluted the 2 N Stock solution with double distilled water 1:1).

**Standard Stock solution Preparation**

Bovine serum albumin crystals were used at a concentration of 25 mg / 5 ml IN NaOH. Different dilutions in the range of 0.25 - 2.5 mg/ml were prepared from this stock solution and the alkaline mixture and Folin-ciocalteau's reagent were added as in the case of tissue samples.

A blank was prepared with 1ml IN NaOH and followed the same way as the sample treated above. Then all the test tubes were kept for 30 minutes at room temperature and the optical density of the blue colour developed was measured against the blank at 660 nm by Spectrophotometer (Thermospectronic, Genesys-5; UV/VIS spectrophotometer)

**3.7.3. Total Carbohydrates**:

The Phenol sulphuric acid method of Dubois *et al* (1956) was followed to estimate the total carbohydrates in the sample.

The supernatant obtained during protein estimation procedure was used for the carbohydrate estimation.1ml of the supernatant of muscle, ovary and blood serum was taken separately. 1 ml of 5 % phenol (5.5 ml of 90% liquid phenol added to 94.5 ml water) was added and mixed well. In the case of liver tissue, 0.5 ml of the supernatant was made up to 1ml with saturated solution of benzoic acid. To the above solution 1 ml of 5 % phenol solution was added. Then to this solution 5ml of concentrated $H_2SO_4$ was added rapidly and carefully to each tube and mixed well using a cyclomixer.

**Standard Stock Solution preparation**:

D-Glucose (concentration – 20 mg /100ml saturated solution of benzoic acid) was used as stock solution. Different dilutions of the working solution with the concentration of glucose ranging from 10-100µg/ml were prepared. 2 ml of 5% phenol was prepared and used as blank solution.

All the tubes were kept for 30 minutes at room temperature and the optical density of the orange colour developed was measured at a wavelength of 490 nm by a spectrophotometer ( Thermospectronic, Genesys-5, UV/VIS spectrophotometer)

### 3.7.4. Estimation of Total Lipids

To estimate total lipids in the tissue samples, Barnes and Blackstock method (1973) was used. Wet tissue weighing 10 mg of muscle and ovary, 5mg liver samples were separately homogenized well in 1 ml of chloroform : methanol (2 : 1v/v) and kept overnight at 4°C for complete extraction. 0.1 ml of serum was thoroughly mixed with 1 ml 2: 1 v/v chloroform: methanol and left overnight in the refrigerator.

The mixture taken in 2ml eppendorf tubes were then centrifuged for 15minutes at 3000 rpm. Then the supernatant transferred to clean glass tubes. From the above supernatant 0.5 ml of lipid extract was taken separately for each tissue and dried *in vacuum* over silica gel in a dessicator

To each dried sample, 0.5 ml of conc. $H_2SO_4$ was added and shaken the glass tubes well. Then the glass tubes were plugged with cotton and heated at 100°C in a boiling water bath for 10 minutes. After boiling the glass tubes were rapidly cooled to room temperature under running tap water. To 0.1 ml of the above acid digest, 2.5 ml of phosphovanillin reagent was added and mixed well in a cyclomixer.

**Standard stock solution preparation**

80 mg of cholesterol dissolved in 100 ml of chloroform: methanol (2.1 v/v) mixture (equivalent to 100 mg of total lipid in 100ml ( 2 : 1 v/v) chloroform-methanol mixture). Working solutions of different concentrations were prepared from the stock solution in the range of 50 - 500µg / 0.5 ml and the procedure adapted to tissue samples was followed. 0.5 ml of 2: 1 v/v chloroform- methanol mixture was treated as blank.

All tubes were kept at room temperature for 30 minutes and then the intensity of pinkish red color developed was measured against blank at 520 nm with spectrophotometer (Thermospectronic Genesys-5, UV/VIS spectrophotometer).

### 3.7.5. Estimation of Total Cholesterol

To analyze the total cholesterol content of the sample Henly' s method ( 1957) as given by Varley ( 1976) was used.

Wet tissue samples (muscle, liver & ovary) of 25 mg each were weighed accurately and homogenized well with 10 ml of ferric chloride- acetic acid reagent ( 0.05 % solution $FeCl_3$ .6 $H_20$ in glacial acetic acid ) and placed at 4°C and left over night in refrigeration for the protein to flocculate. For the blood samples 0.1 ml of serum was mixed well with 10 ml of $FeCl_3$ - $CH_3COOH$ reagent and left over night in the refrigerator. All the samples were centrifuged for 20 minutes at 3000rpm and 5 ml of the supernatant from each tube was transferred to clean dry glass tubes. 3 ml of concentrated $H_2SO_4$ was added to all the tubes and thoroughly mixed in a cyclomixer.

**Standard stock solution preparation**

A volume of 100 mg of cholesterol dissolved in 100 ml of glacial acetic acid to get a concentration of 1 mg/1ml. Working solution was prepared afresh by mixing 1 ml of stock solution with 24 ml $FeCl_3$ - $CH_3COOH$ reagent. Different dilutions of the solution with the concentration of cholesterol ranging 20-200ug/5ml were taken and treated with 3 ml of concentrated $H_2SO_4$ as in the case of tissue samples. 5 ml of $FeCl_3$ – $CH_3COOH$ reagent was treated as the blank solution.

All the tubes were allowed to stand for 30 minutes at room temperature. The intensity of reddish brown colour developed was measured against the blank at 560nm by spectrophotometer.

### 3.7.6. Ash Content

A pre- weighed amount of oven dried powdered tissue sample was ignited in a silica crucible for 5 hrs at 600°C in a muffle furnace , till all the organic matter was burned out leaving no carbon residue. The ignited content was weighed and the difference in weight gave the ash content of the tissue ( AOAC, 1990). The percentage ash content of the tissue was calculated as follows.

$$\text{Ash \% mg} = \frac{\text{Ash weight}}{\text{Dry weight of tissue}} \times 100$$

### 3. 7.7. Estimation of total Carotenoids

The method described by Olson (1979) was adopted to estimate the total amount of carotenoids in different tissues and blood serum. About 1 gm. of the fresh tissue (muscle, liver and ovary) was weighed on a small piece of aluminium foil and placed in labeled clean dry screw capped 10 ml vials, containing 2. 5 gm of an anhydrous sodium sulphate. The tissues were gently mashed using a glass rod. 5 ml of chloroform was added to each borosil vial to extract all the carotenoids from the sample. For the blood samples, 1 ml of the serum was mixed well with 5 ml of chloroform in a clean screw capped vial. All the vials were sealed and kept overnight at 0°C in the dark. The blank contained only 5ml of chloroform.

An aliquot of 0.3 ml chloroform extract form each vial was diluted to 3ml with absolute ethanol. The blank was also treated in similar manner. The optical density was read with spectrophotometer at 380, 450, 475 and 500 nm. The wave length with maximum absorption (450 nm) was used for calculation.

The total carotenoid content in µg carotenoid / gm in the muscle, liver and ovary was calculated as follows.

$$\text{Total carotenoids} = \frac{\text{Absorption at 450nm X dilution factor}}{\text{0.25 X sample weight in gm}}$$

Here; dilution factor = 50

Extinction co-efficient = 0.25

In the case of serum, total carotenoids, as µg / ml was calculated as follows.

$$\text{Total carotenoids} = \frac{\text{Absorption at 450 mm x dilution factor (50)}}{\text{0.25 X sample volume (ml)}}$$

When adequate amount of tissues were not available from a single specimen, tissues from different specimens belonging to the same stage of maturity and size group were pooled together to make a sample for analysis. In the case of blood samples when the collected blood was not sufficient enough to different parameter studies pooling of the blood samples of same maturity stages was practiced. All the biochemical analyses were carried out for five replications.

Standard graphs were plotted with the concentration of each biochemical parameter in different dilutions of the working standard solution in the X-axis and the optical density in the Y-axis. The concentrations of different parameters in the sample were calculated by comparing the optical density obtained for the sample with the values in the standard graph and also using the following formula.

Concentration in mg/100 mg wet tissue

$$= \frac{\text{(OD. of the sample - OD. of the blank)}}{\text{(O.D. of the standard - O.D. of the blank)}} \text{ X Concentration of standard X } \frac{100}{\text{sample weight (mg)}}$$

Concentration in mg/ 100 ml blood serum

$$= \frac{\text{(O.D of the sample - OD. of the blank)}}{\text{(O.D. of the standard - O.D of the blank}} \text{ X Concentration of standard X } \frac{100}{\text{sample volume (ml)}}$$

# Results

# 4. RESULTS

## 4.1 Reproductive biology

Mature ovaries and testes are simple to distinguish. Ovaries are tubular and normally pink, yellow or orange. Ovaries seem to be surrounded by a clear bag. This can be seen by picking at them with forceps.

### 4.1.1 Morphology

The reproductive system of females of *E. diacanthus* includes a pair of ovaries, continued into an oviduct and ends in genital pore. The ovaries are paired egg sacs located behind the stomach and duodenum, below the swim bladder, and just above the intestine, and connected to it by mesenteries. Each ovary consists of a hollow sac. The right and left lobes are usually unequal in size. Right ovarian lobe is relatively larger than the left, both of which join posteriorly and descend as an oviduct to open in the genital pore immediately behind the anus. The urinary bladder is closely bound to the posterior face of the common oviduct. Supporting mesenteries continue forward from the anterior end of each gonad as ligaments that join a complex of ligaments and mesenteries at the anterior end of swim bladder.

*E. diacanthus* ovary is of the cystovarian type in which matured eggs will be released into the ovarian cavity during the ovulation; then ova will pass through oviduct on their way to go out at the genital pore. The genital pore is seen as a smaller pore behind the anus; this pore will be bigger and pinkish during spawning season. The wall of the gonad is covered externally with a peritoneal layer. The tunica albuginea has an intermixture of longitudinal, oblique and circular muscle fibers.

## 4.1. 2 Morphological Classification of maturity stages

### Stage I

Stage I of maturity stage in *E. diacanthus* is defined as an ovary that shows no signs of prior spawning known as Immature. The ovary is relatively small, translucent and white pinkish in colour. The ovary contains oocytes in stage 1 and stage 2. Atretic bodies are not present in this maturity stage. (Plate.3)

### Stage II

Mature resting female / maturing female stage II of *E. diacanthus* is defined as an ovarian stage that had undergone extensive vitellogenesis and recovered into resting state. The ovary is larger than the previous stage and white brownish in colour. Oocytes in stages 1, 2 and sometimes 3 are present, with those in stage 2 dominating the ovary (Plate. 3)

### Stage III

Stage III is defined as the ovarian stage in which active vitellogenesis is in preparation for spawning, in the mature active female / Ripe female. The ovary occupies 2/3 of the body cavity and is yellowish in colour. Oocytes are in stages 1, 2, 3 and 4 with stage 3 oocytes dominating during early development of this stage. Stage 4 oocytes then dominate the ovary until spawning taken place. Ovary may remain in stage III for several weeks until spawning conditions are present. Oogonia and stage1 oocytes are uncommon in stage III ovaries. Atretic bodies are usually present but they are obscured by the extensive oocyte development. (Plate. 3 and Plate. 4):

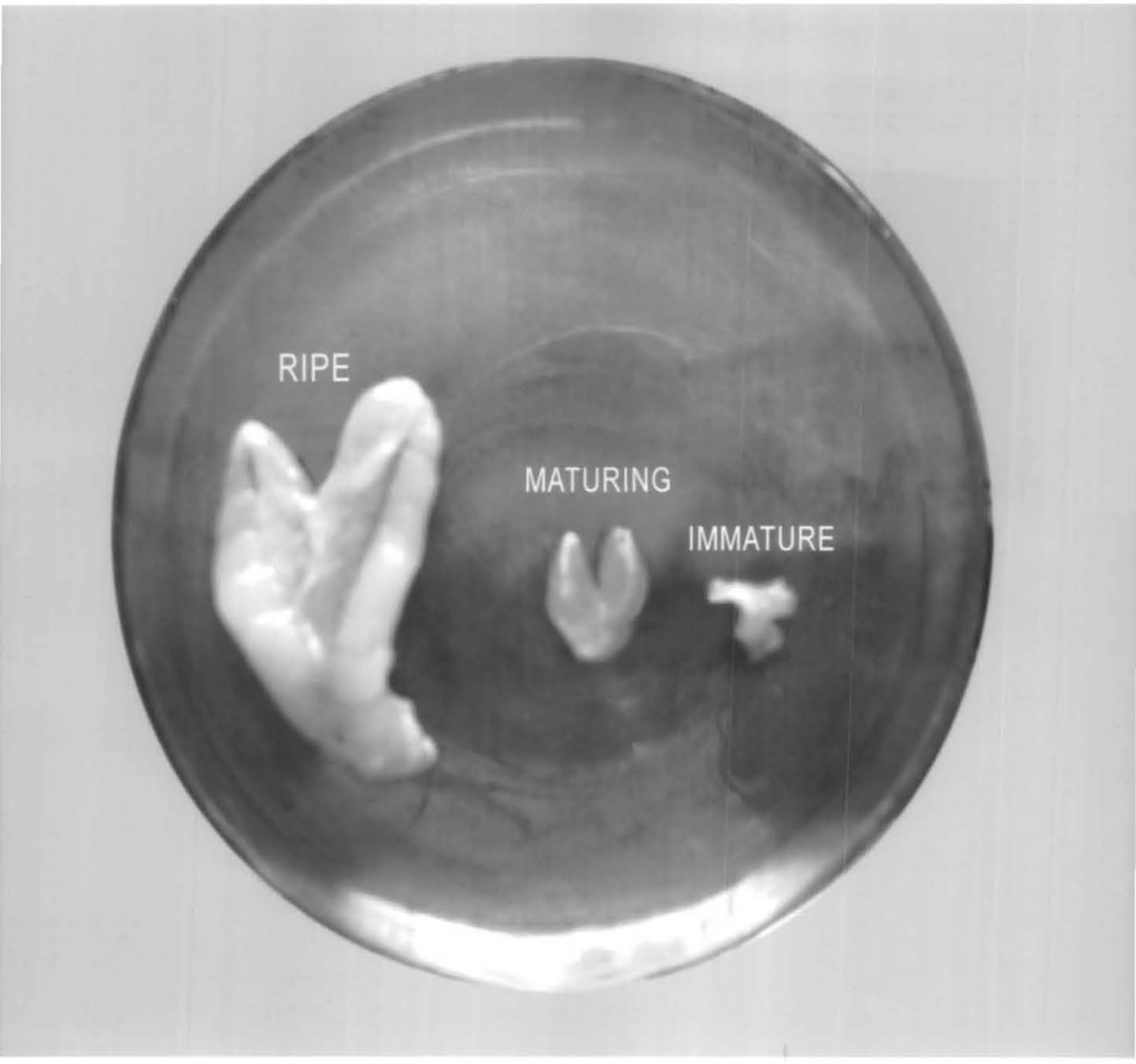



**Plate 3. Morphology of various maturity stages of female E. *diacanthus***

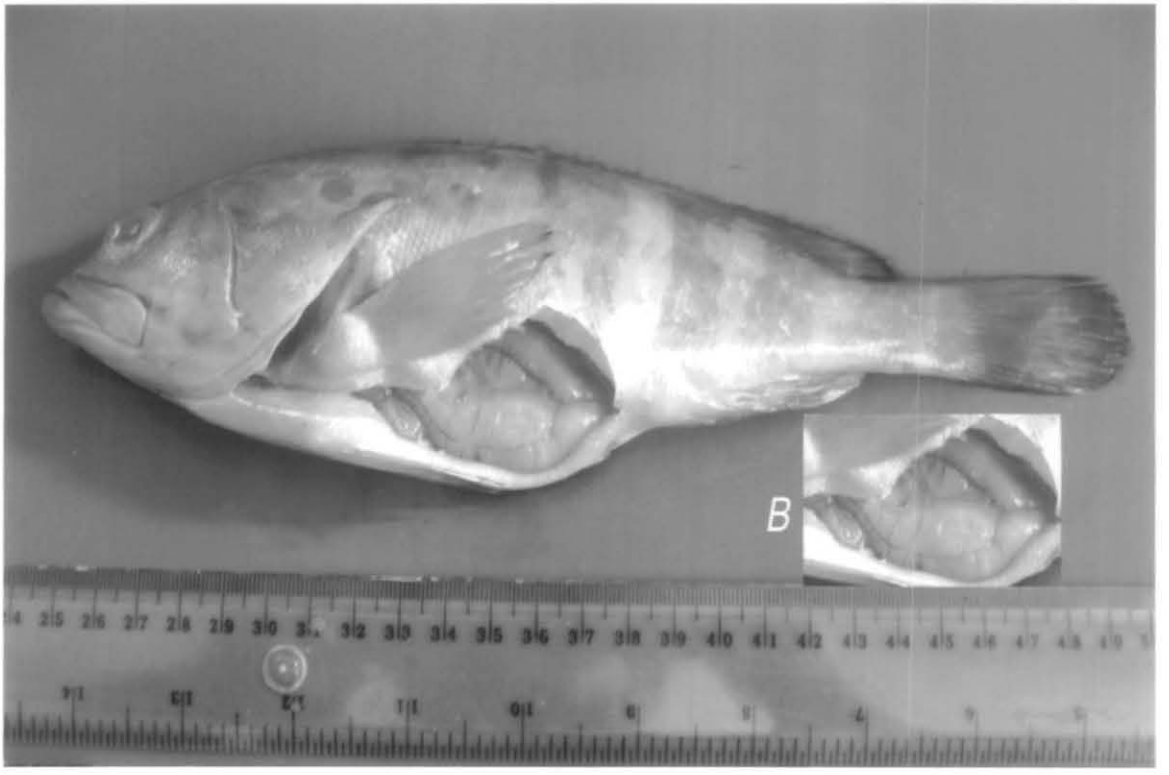



Plate 4. *E. diacanthus* with ripe ovary
B - exposed portion of ripe ovary

## 4.1. 3 Distribution of ova in the ovary

**Stage I**

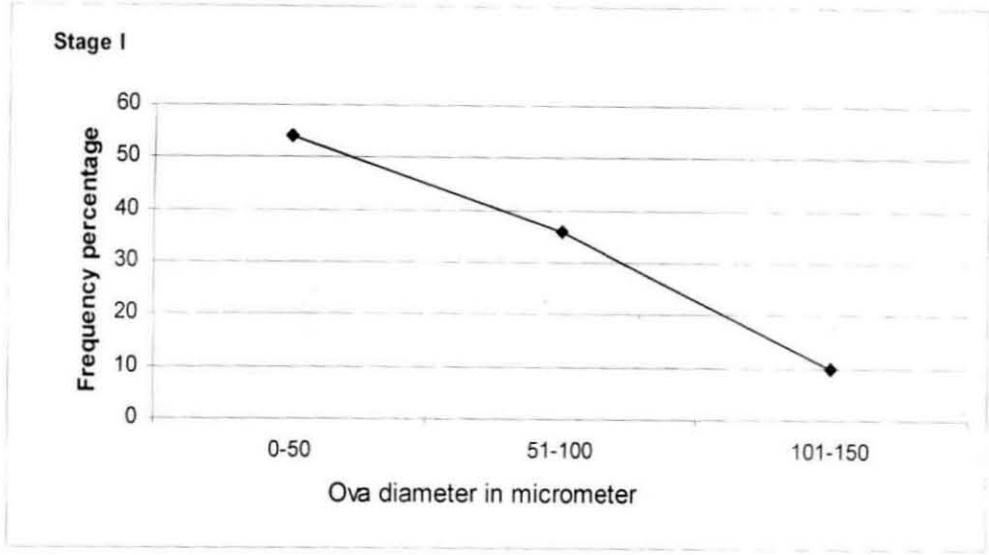
In this stage, the ovaries appear very small and transparent. They occupy less than one third of the body cavity. Majority of the ova in this stage are between 0 -150µm size range where in the majority of oocytes are in 0 - 50 \µm size. The ova are immature with modes at 0 - 50 µm and 51 - 100 µm diameter (Fig.2 a).

**Stage II**

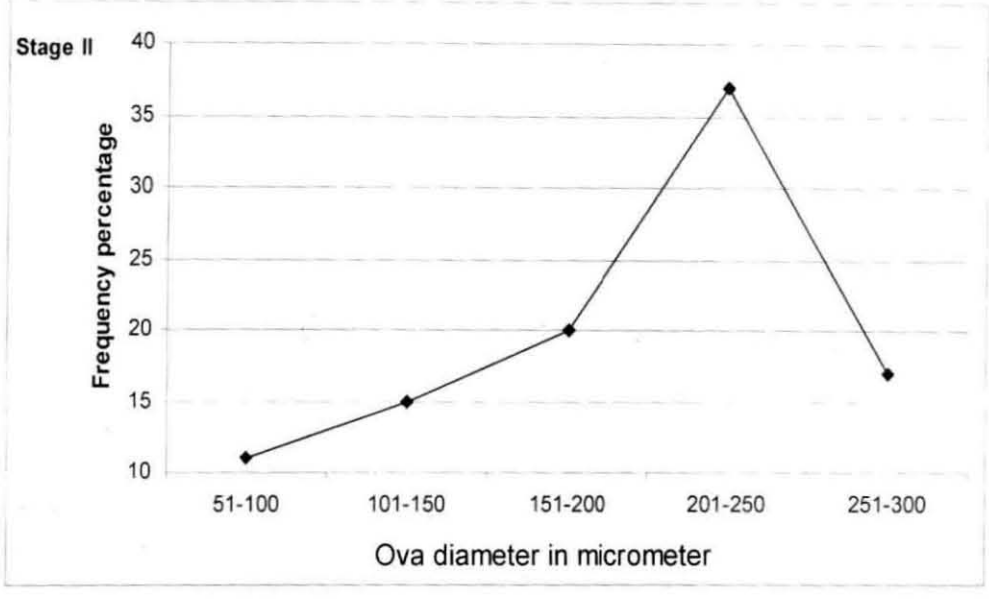
Ovaries in this stage are slightly larger, occupying more than one-third of body cavity. Maturing group of ova with a mode shifting to 201 - 250 µm are dominant. The size range of the ova at this stage was from 51 to 300 µm ( Fig. 2 b).

**Stage III**

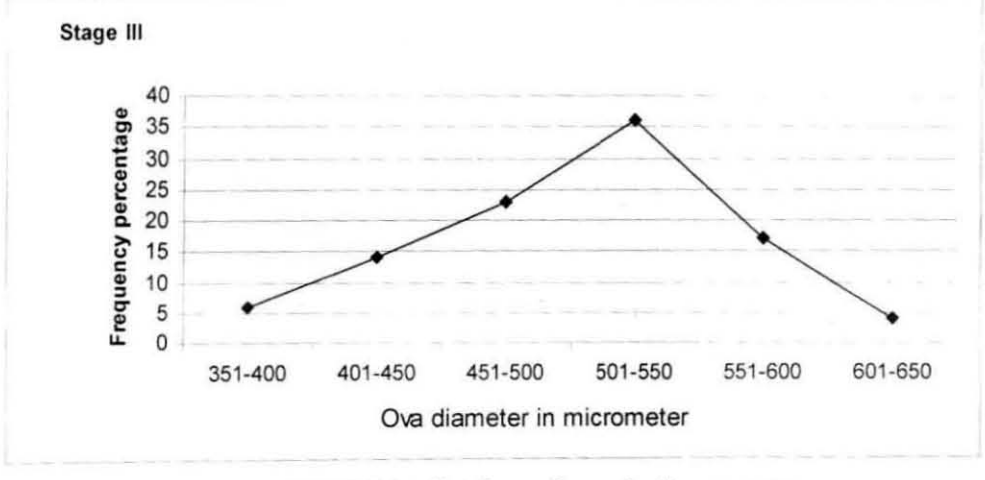
The ovaries occupy the entire body cavity. Largest group of ova with a mode at 501 - 550 µm and with secondary modes at 351 - 400 µm, 401 - 450 µm, 451 - 500 µm, 551 - 600 µm and 601- 650 µm. In the stage III of *E. diacanthus* ovary, 650 µm was the maximum ova diameter recorded (Fig. 2 c).




(a)




(b)




(c)

Fig.2 Distribution of ova in the ovary

### 4.1.4 Gonado-somatic index (GSI)

In the present study, GSI values of *E. diacanthus* ovary have showed correlation with the maturation of gonads (Fig.3). The immature ovaries in the maturity stage I showed a GSI value of 0.062 %, the maturing ovaries in the stage II the value is 0.234 % and in the ripe stage ovaries of the maturity stage III, the value is 3.064 %. Highest GSI value was observed in stage III of gonadal maturation. From the Fig.3 it is evident that in *E. diacanthus,* the increase of ovary weight is associated with the progress of maturity of the ovary. Table 5 shows the GSI values.

### 4.1. 5 Hepato-somatic index (HSI)

The hepato somatic index, which is the percentage of liver to the body weight, was found to increase gradually from immature to ripe stages in the female *E. diacanthus*. The hepato somatic index was highest during the ripe condition of the ovary (stage III).

The hepato somatic index (HSI) in female *E. diacanthus* at stage I and stage II were 1.38 % and 2.062 % respectively. Highest hepato somatic index in the female *E. diacanthus* was noticed in stage III (2.549%) (Table 5 and Fig. 4).

### 4.1.6 Condition factor (K)

In the present study, condition factor in *E. diacanthus* was in the range of 1.15 – 1.61. Highest condition factor (1.61) was observed in stage III of gonadal maturation. (Table 5 and Fig. 5).

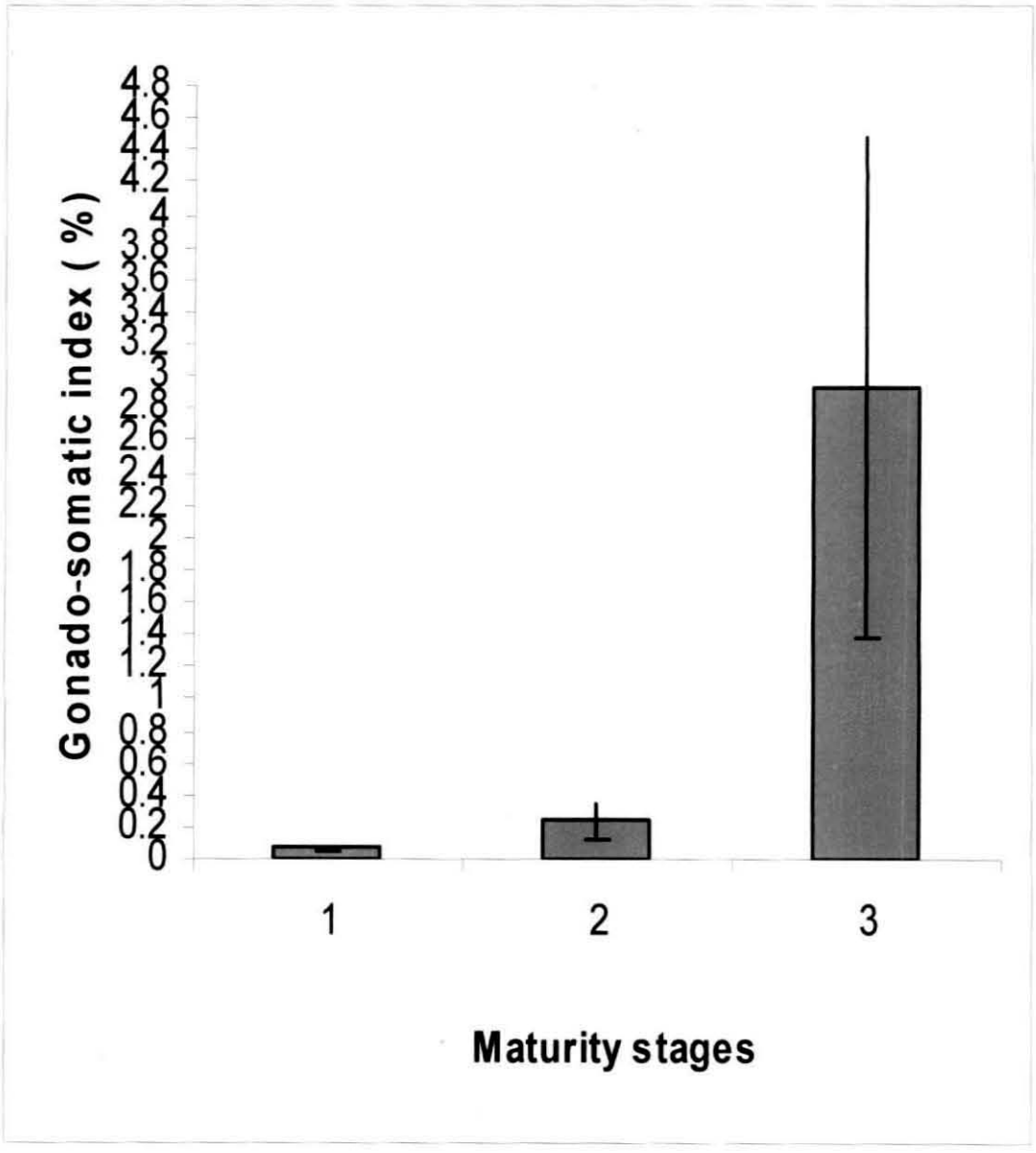



Fig. 3 Trends in the GSI values with the maturation of gonads (Mean ± SD)

Table 5

Variations in the GSI, HSI and Condition factor (K) values with maturation of gonads

| Maturity stage | GSI (%) | HSI (%) | K |
|---|---|---|---|
| Stage I | 0.062 ± 0.004 | 1.38 ± 0.02 | 1.15 ± 0.01 |
| Stage II | 0.234 ± 0.105 | 2.062 ± 0.205 | 1.4 ± 0.05 |
| Stage III | 3.065 ± 1.550 | 2.549 ± 1.03 | 1.61± 0.85 |

(Mean ± SD)

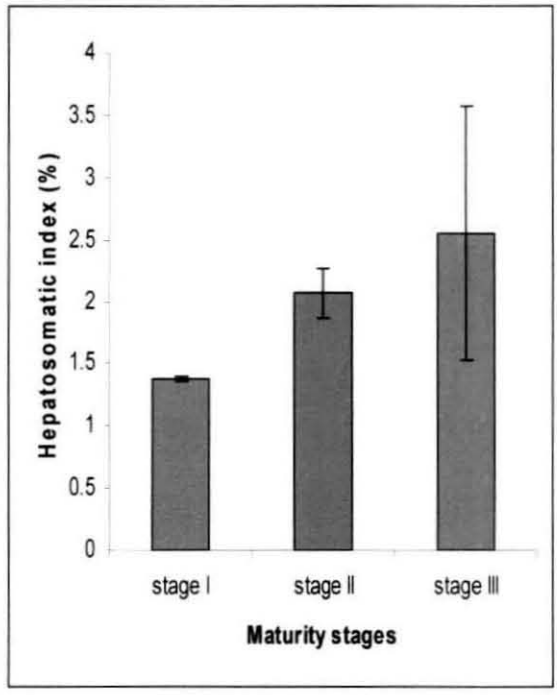



Fig 4. Trends in the HSI values with the maturation of gonads (Mean ± SD)

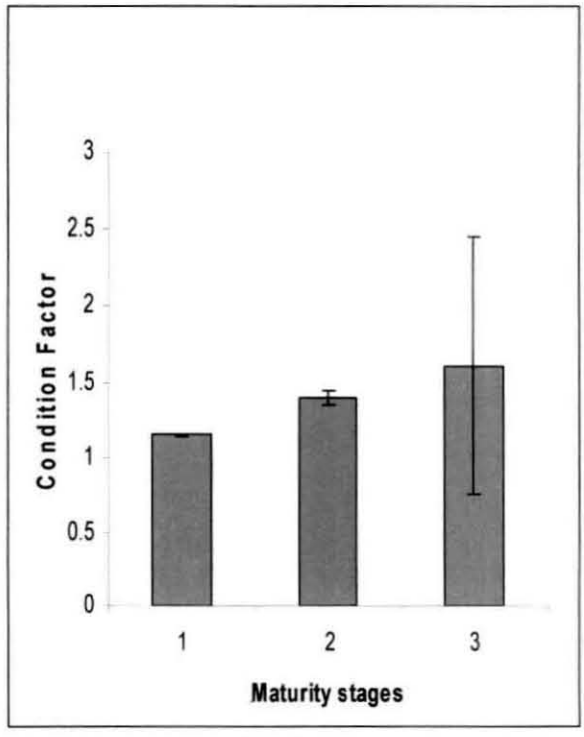



Fig. 5 Trends in Condition factor with the maturation of gonads (Mean ± SD)

### 4.1.7 Fecundity

Fecundity of fishes is usually determined from the number of ova of the mature group in the ovary. The fecundity of *E. diacanthus*, is determined in the present study from the examination of 25 specimens, and the details are given in the Table 6. The fecundity of *E. diacanthus*, varies from 13.1x $10^3$ to 145.7 x $10^3$ with an average of 75, 547ova.

**Relationship between fecundity and weight of ovary**

The number of eggs is plotted against the weight of ovary in a scatter diagram (Fig. 6a). It is found that fecundity generally increases with increase in weight of the ovary. The relationship between fecundity and gonad weight in *E. diacanthus* was linear (Fig. 6 a). The regression of fecundity on gonad weight can be expressed as F= 9577.9GW+32385 and the $r^2$ value was found to be 0.5841. The values indicate the correlation was significant

**Relationship between fecundity and weight of fish**

The observed values of fecundity for 25 specimens are plotted against the weight of fish in Fig.6 b. The relationship between fecundity and weight of fish in female *E. diacanthus* was linear and it shows a gradual increase of fecundity with total weight increase. The regression equation of fecundity on total weight can be expressed as F = 11.586TW + 72163 (F= fecundity; TW = Total weight) and $r^2$ value was found to be 0.0115.

**Relationship between fecundity and total length**

The number of eggs produced by individuals of *E. diacanthus* is plotted against the length of fish (Fig.6 c). In the present study fecundity has showed low correlation coefficient with the total length of the fish. The regression of fecundity and total length can be expressed as F = 677.14TL + 56947 (TL = Total length) and $r^2$ value was 0.0217.

**Relationship between fecundity and standard length**

The relation between fecundity and standard length of fish was tested by plotting the observed values in a scatter diagram (Fig. 6 d). In *E. diacanthus* it shows a linear regression. The regression of fecundity and standard length can be expressed as F = 556.21SL + 63141 (SL = Standard length) and $r^2$ value was 0.01.

Table 6

Fecundity estimations and morphometric measurements of the ripe female, *E. diacanthus* used for the fecundity studies (N = 25).

| Total length(cm) | Standard length(cm) | Total weight (gm) | Gonad weight (gm) | Fecundity |
|---|---|---|---|---|
| 36.2 | 30 | 540 | 5 | 67419 |
| 43.9 | 35.5 | 1000 | 10.02 | 78385 |
| 34.4 | 20.3 | 204 | 7.93 | 96418 |
| 23.5 | 19.4 | 174 | 7.8 | 145755 |
| 31.2 | 25.8 | 435 | 7 | 121301 |
| 22.5 | 17.2 | 148 | 6.7 | 107400 |
| 49.1 | 41.3 | 1475 | 2.71512 | 71409 |
| 24.6 | 20.3 | 188 | 0.75 | 13125 |
| 22.7 | 18.7 | 133 | 3.35 | 57533 |
| 21.9 | 18 | 137 | 3 | 51810 |
| 20.3 | 16.3 | 109 | 4.15 | 74907 |
| 19.4 | 15.8 | 86 | 2.42 | 39010 |
| 23.6 | 19.3 | 158 | 6.05 | 99341 |
| 20.1 | 16.7 | 98 | 3.6 | 65340 |
| 25.7 | 21 | 197 | 1.47 | 29458 |
| 24.1 | 19.7 | 171 | 7.12 | 104165 |
| 21.9 | 17.9 | 148 | 1.01 | 12049 |
| 35.6 | 29.7 | 190 | 5.103 | 67,409 |
| 24.8 | 20.3 | 210 | 0.985 | 73,125 |
| 25.3 | 21.5 | 197 | 0.716 | 32,458 |
| 26.1 | 22.6 | 225 | 1.027 | 85,786 |
| 28.4 | 23.7 | 510 | 6.841 | 125,049 |
| 30.9 | 24.3 | 195 | 8.112 | 101,165 |
| 24.5 | 19.8 | 200 | 4.23 | 89,879 |
| 26 | 22.5 | 172 | 3.471 | 78,967 |

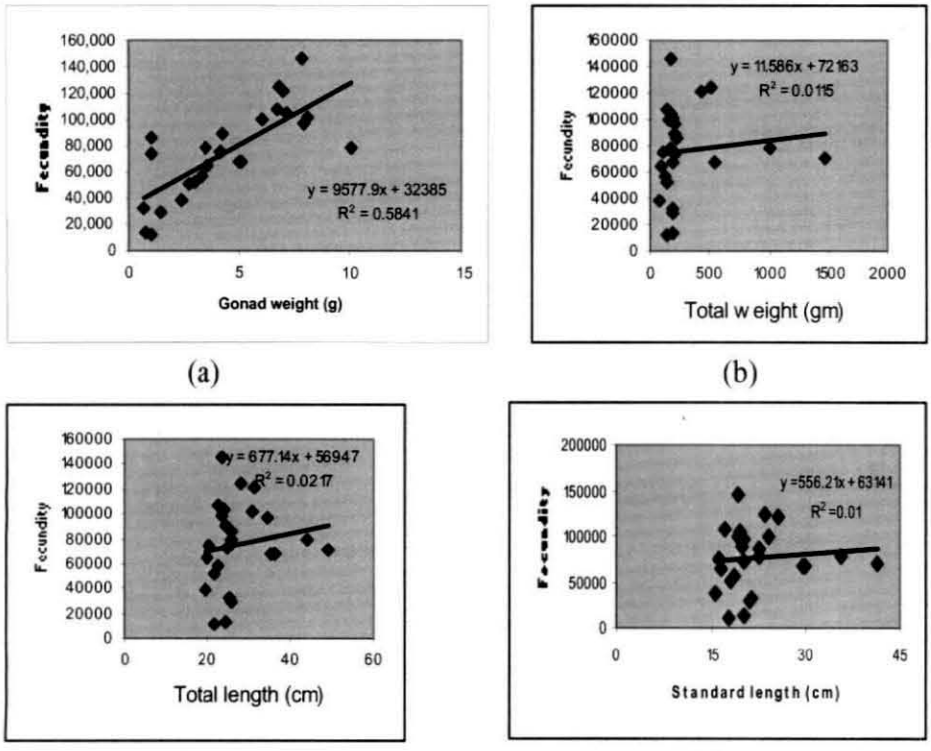



Fig.6 Fecundity relation ship with total length, standard length, total weight and gonad weight.

# 4.2 Histology of Oocytes and Hepatocytes.

Reproduction is a dynamic metabolic activity in most fishes and it involves sequential changes in its germ cells. The pattern of these changes in the gonads is typical for each species. To clearly understand the physiology of fish during reproduction, the study of the seasonal developmental changes of gonads through both macroscopic and microscopic observation is necessary. In the case of hermaphroditic fishes macroscopic observation may not provide the correct information of the germ cell development during gonadal maturation and has its own limitation. Hence, microscopic observation is considered as important for detailed information on the reproductive mechanism of such fishes. Histological observation will provide information on internal changes in the germ cell and the ultra structure studies will provide more detailed information on the cytological and cytochemical changes in the gonadal maturation. Hence, an attempt is made in this direction to study the maturation cycle of *E. diacanthus.*

## 4.2.1 Microscopic studies

**Oogenesis**

The description was made on the basis of gonad sections of *E. diacanthus.* The study was followed vide the description by Moe (1969) and adopted by Tessy (1994).

**Stage 1- Immature**

In this stage the diameter of the oocyte ranged between 17 and 50 µm. The cytoplasm becomes strongly basophilic. A thin follicular layer surrounds the oocyte at this stage. The ovary contains stage 1 and stage 2 oocytes (Plate. 5).Chromatin nucleolus stage oocytes are more abundant than perinucleolus stage oocytes. Primary stage oogonial cells observed in this stage are embedded in the ovigerous tissue and usually found along the periphery of the ovarian lamellae.

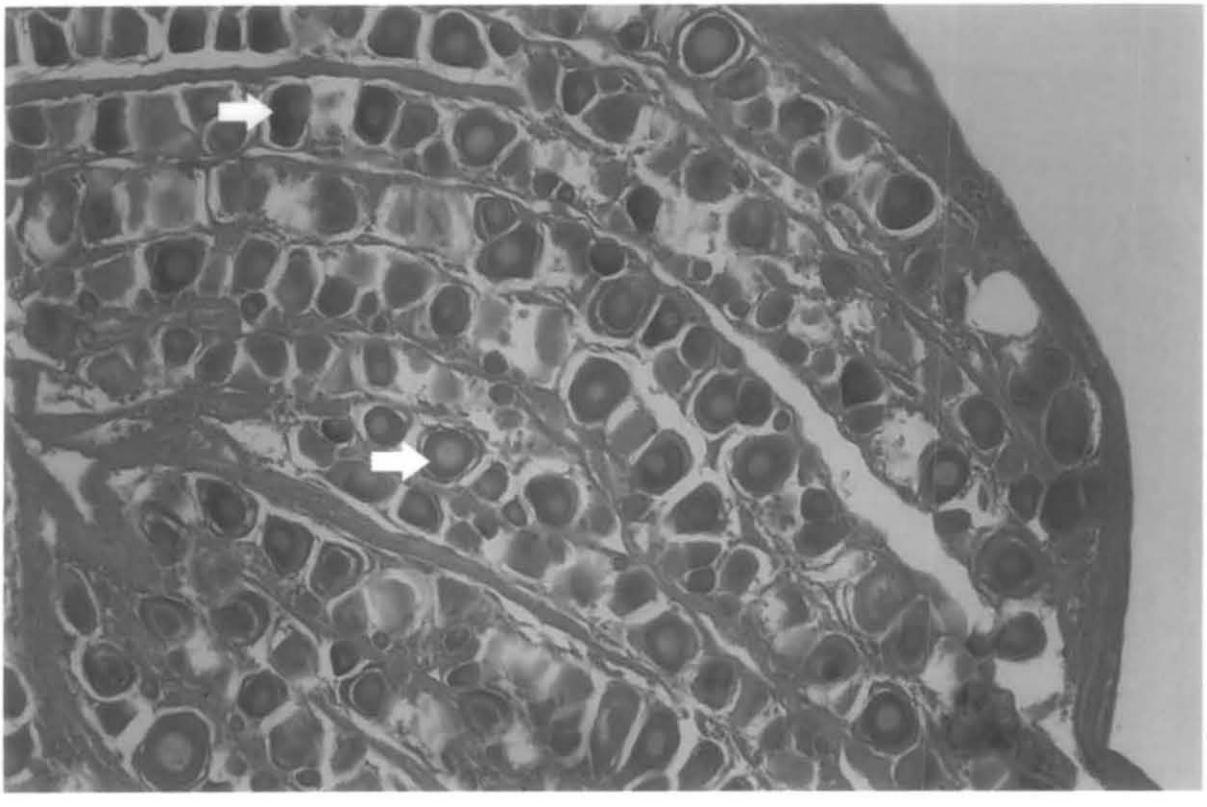


*Plate 5. Light micrograph of gonad development stage I, immature ovary of female E. diacanthus. Upper arrow indicates stage 1 and lower arrow indicates stage 2 oocytes. (10X)*

**Stage 2- Maturing**

During this stage the oocyte diameter size varied from 60 to 250 µm. This stage occurs before vitellogenesis. The ovary contains stage 3 oocytes abundantly. The cytoplasm is strongly and evenly basophilic. A thin follicular layer surrounds the oocyte (Plate.6).

**Stage 3- Mature active / Ripe**

The size range of oocyte diameter in this stage was from 80 µm to 520 µm. The oocyte expands generally and regains its rotundity. The nucleus also increases in relative to its size. The ovary contains early and late vitellogenic oocytes. The stage 4 oocytes are abundant in the ovary (Plate.7)

The vitellogenic oocytes continue to expand and reach maximum attainable size before ovulation.The nucleus is well defined in early stage 4 (Plate. 8). Yolk vesicles are prominent and usually surround the nucleus in early stage 4 and coalesce towards the centre, when nucleus loses its definition. These yolk vesicles are usually evident in late stage 4 near the oocyte periphery. Acidophilic yolk globules largely replace the basophilic cytoplasm in early stage 4 and become large and well developed in mid stage. The yolk globules coalesce in late stage 4 and present a smooth acidophilic appearance.

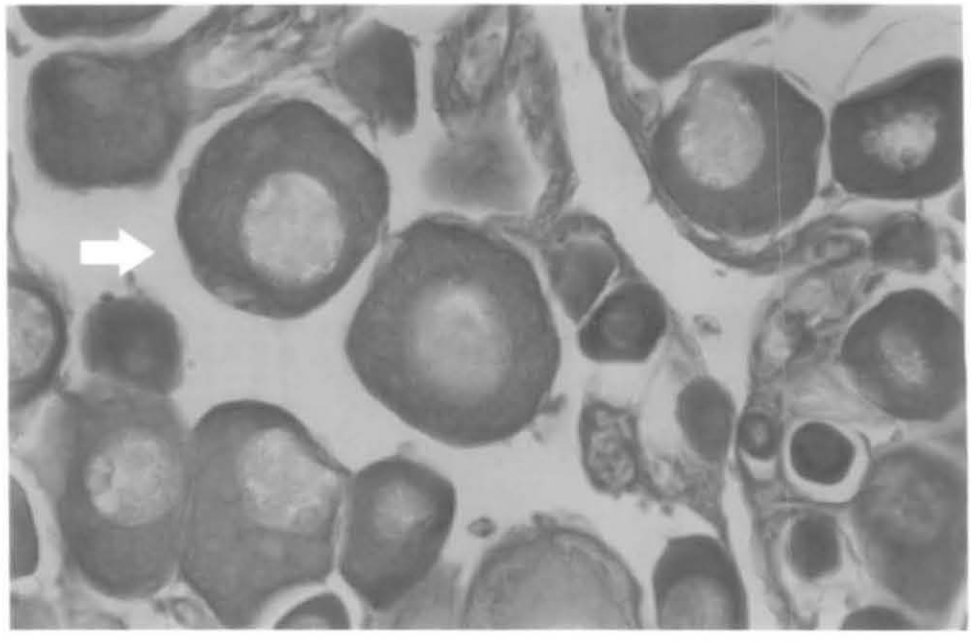


Plate 6. Light micrograph of maturing ovary of female *E. diacanthus*. Above arrow indicates stage 3 oocytes. (40X)

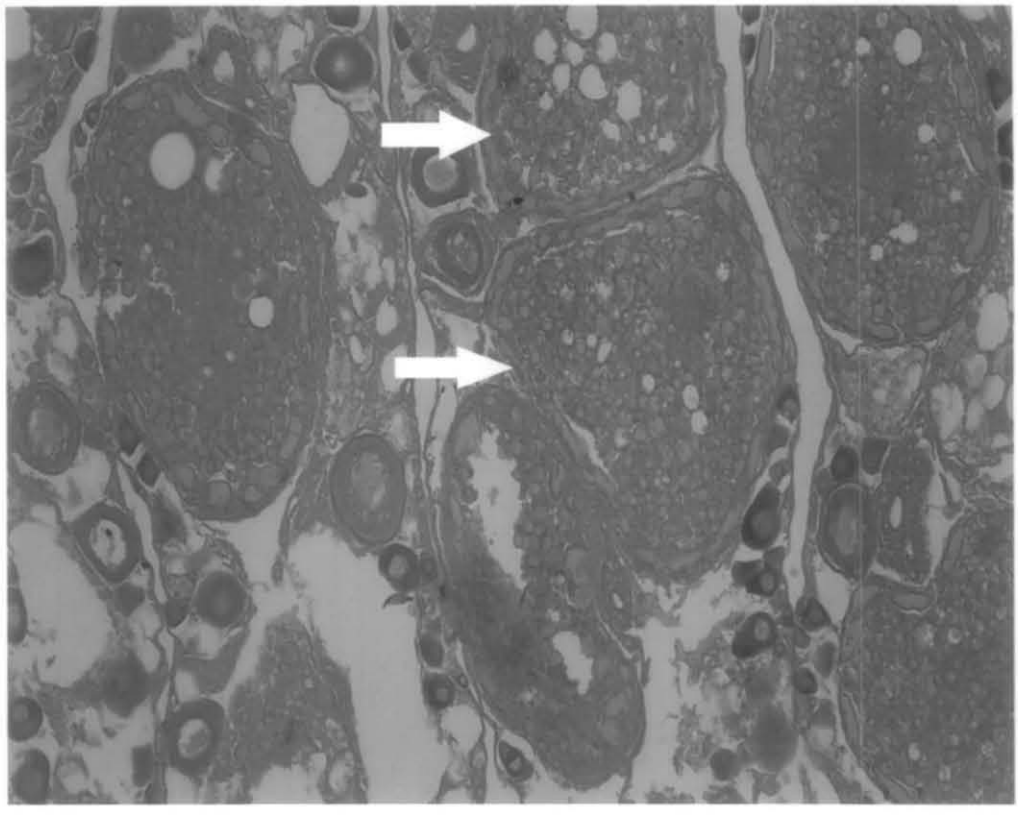


Plate 7. Light micrograph of female *E. diacanthus* ripe ovary. Above arrow indicates early vitellogenic oocytes. Below arrow indicates late vitellogenic oocytes.(10X)

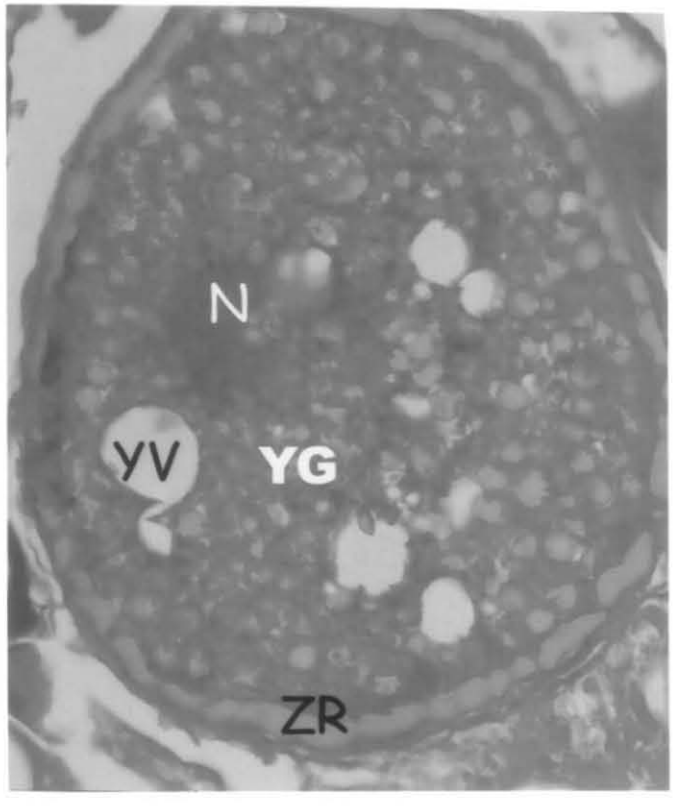



Plate 8. Light micrograph of late vitellogenic oocyte which is showing migration of nucleus to the periphery of the oocyte. (20X) ,N -Nucleus, YV- Yolk vesicle, YG- Yolk globule, ZR- Zona radiata.

## 4.2.2 Ultra structural studies of oocytes

### Primary stage oogonia

### Cytology

Nucleus is large and oval in shape. Nucleoplasm appears electron dense, containing small clumps of chromatin more near the nuclear envelope. Oogonia have nucleus with a distinct envelop and cytoplasm has polar distribution of cell organelles. In *E. diacanthus* primary stage oogonia has showed mitochondria associated with cement and nuages. Large nucleus to cell ratio was observed. (Plate. 9)

### Cytoplasmic characters

The *E. diacanthus* oogonial cytoplasm contains mitochondria, free ribosomes and scant endo plasmic reticulum. Golgi complex is not distinctly seen. Mitochondria are round and associated with cement and nuages. Few granulocytes observed in the periphery of oogonial cytoplasm. The electron dense nuages are observed scattered in the cytoplasm.

### Chromatin nucleolus stage (Stage I –Immature)

### Cytology

The roughly spherical, large and eccentrically located nucleus is well developed and occupies greater part of the cell. The nuclear envelope is highly wavy or undulating in nature.

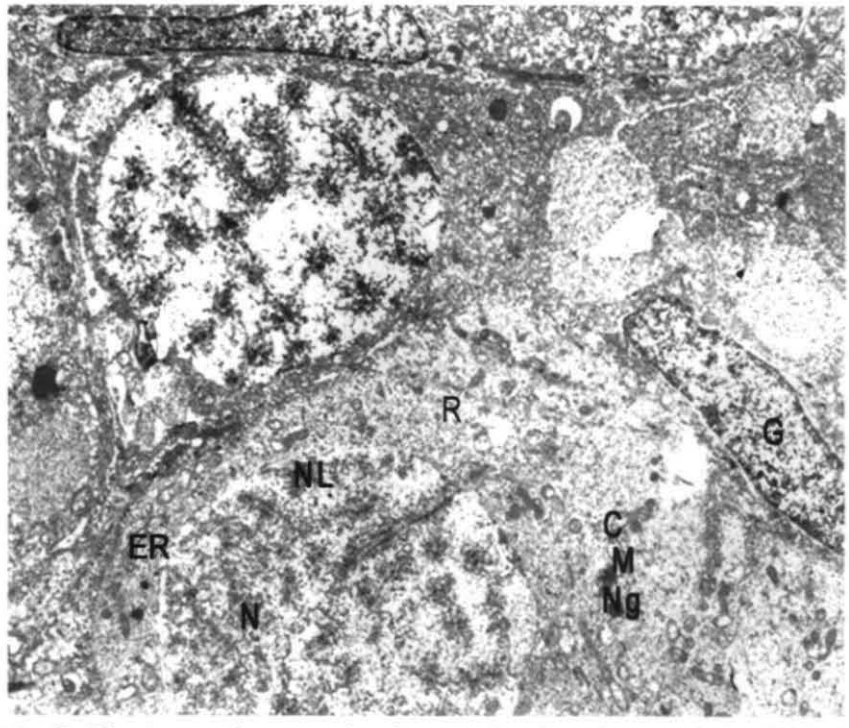



Plate 9. Electron micrograph of oogonia showing nucleus (N) with distinct envelope, nucleolus (NL), Cytoplasm with mitoshondria (M), Cement (C), nuage (Ng), Granulosa cell (G), ribosomes (R) and Endoplasmic Reticulum (ER). (5000X)

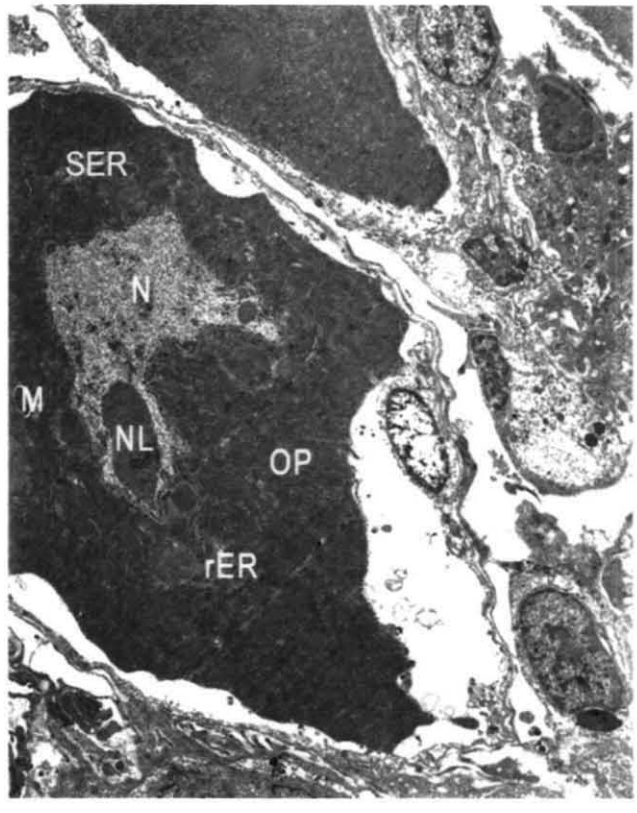



Plate 10. Electron micrograph of chromatin nucleolar oocyte. Note electron dense ooplasm (OP), nucleus (N), nucleolus (NL), mitochondria (M), rough Endoplasmic reticulum (rER), smooth endoplasmic reticulum (SER). (3500X)

**Cytoplasmic characters**

Ooplasm occupies major part of the oocyte after nucleus. This is strongly basophilic and electron dense. Ribosomes are numerous and densely packed in the cytoplasm. Mitochondrial aggregations are conspicuously arranged near the nuclear envelope. (Plate. 10) Few concentric profiles of endoplasmic reticulum observed near the mitochondria. Nuages are scarce.

**Peri nucleolus stage**

**Cytology**

The nucleus increases in size. The nucleoli increase in number. The nuclear envelop runs rather smoothly, occasionally ruptured by nuclear pores. Nuclear envelop is some what irregular in outline. (Plate. 11)

**Cytoplasmic characters**

The cytoplasm is still basophilic and homogenous in appearance. The cytoplasm is increasingly dense. Mitochondria are scattered from the perinuclear to the cortical area, most of them begin to develop as an elongated structures with a clear matrix and many cristae. The ribosomes associated with cristae are not observed. (Plate.11).

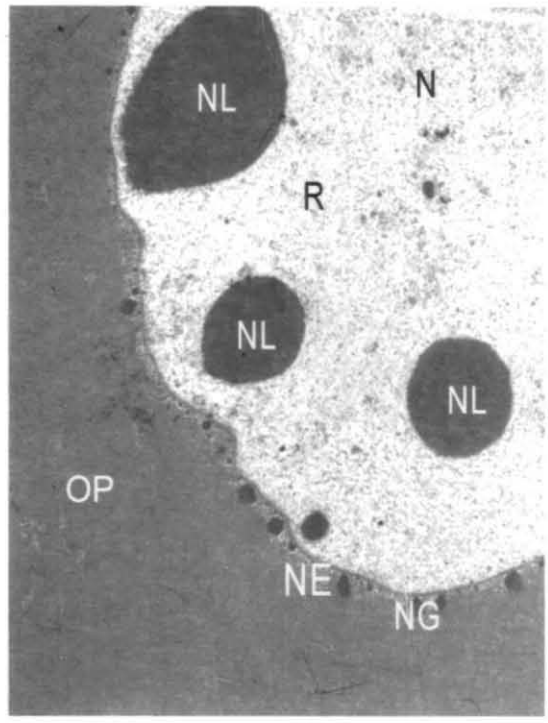



Plate 11. Electron micrograph of perinucleolar oocyte with electron dense ooplasm (OP), nucleus (N), nucleolus (NL), ribosomes (R), nuages (NG), nuclear envelope (NE). (3500x).

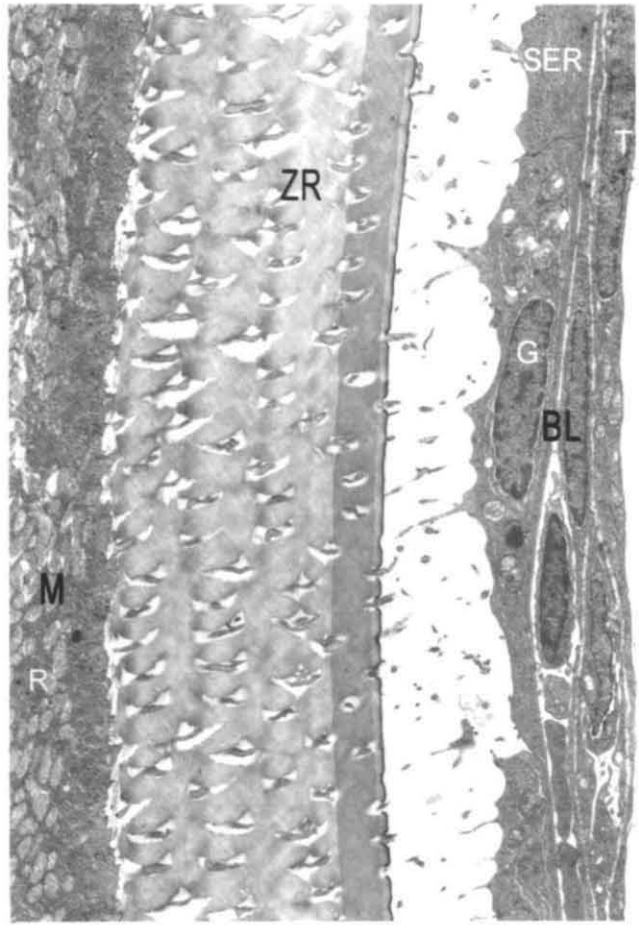



Plate 12. Electron micrograph of maturing ovary with developing thin Zona radiata (ZR), mitochondria (M), basal lamina (BL), granulosa cell (G), thecal cell (T), ribosomes (R), smooth endoplasmic reticulum (SER). (5000x).

**Maturing ovary**

In *E. diacanthus* the oocytes in this stage have showed dense mitochondria aggregation near the zona radiata. The cytoplasmic organelles observed are smooth endoplasmic reticulum and free ribosomes spreading in cytoplasm. Thin zona radiata is present. Few granulose cells are observed near the zona radiata. Basal lamina and thecal cells are also observed (Plate. 12).

**Vitellogenic oocytes (Mature)**

In vitellogenic oocytes mitochondrial aggregation in the cytoplasm is observed. In the early vitellogenic stage, oocytes with lipid droplets were observed (Plate.13) Dense rough endoplasmic reticulum and enlarged mitochondria with tubular cristae are noticed in the cytoplasm (Plate.14). Microvilli are seen in the thick zona radiata (Plate.15). Basal lamina well developed and occupied the middle of granulosa layer and thecal layer (Plate.16). Yolk globules have occupied the most part of the cytoplasm(Plate. 17). Yolk globules have showed zonation of electron dense inner layer and lighter outer layer (Plate.17).

### 4.2.3 Ultra structural studies of hepatocytes in different maturity stages of ovary

**Immature**

Hepatocytes contain a large, round, centrally situated nucleus with a prominent nucleolus. Scattered rough endo plasmic reticulum is observed around the nucleus. Oval shaped mitochondria are present. Dense lipid droplets occupied most of the cytoplasmic area. Dispersed glycogen granules were also observed (Plate.18 and 19).

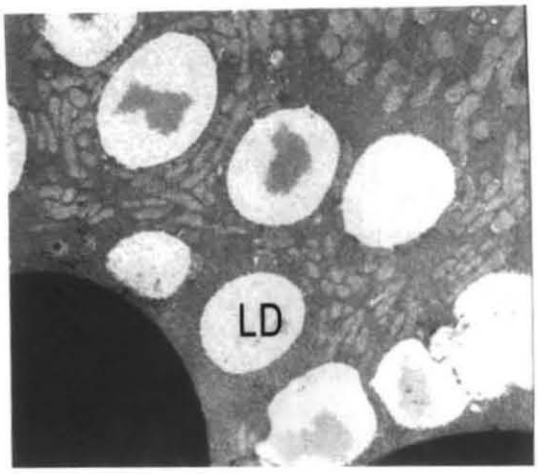



Plate 13. Electron micrograph of early vitellogenic oocytes with lipid droplets (LD). (5000X)

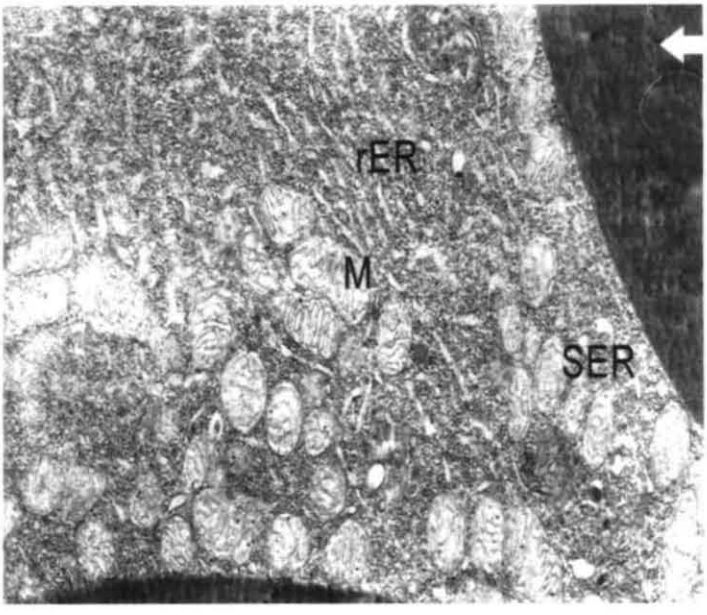



Plate 14. Electron micrograph of late vitellogenic oocytes with dense and enlarged mitochondria (M), dense rough endoplasmic reticulum (rER) and smooth endoplasmic reticulum (SER). (12000x)

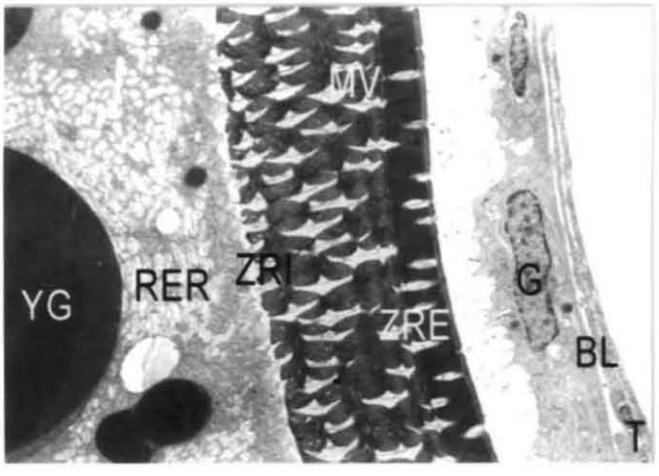



Plate 15. Electron micrograph of vitellogenic oocytes with fully differentiated zona radiata (ZR). zona radiata interna (ZRI), zona radiata externa (ZRE), microvilli (MV), yolk globules (YG), granulosa cell (G), thecal cell (T), basel lamina (BL). (3500X).

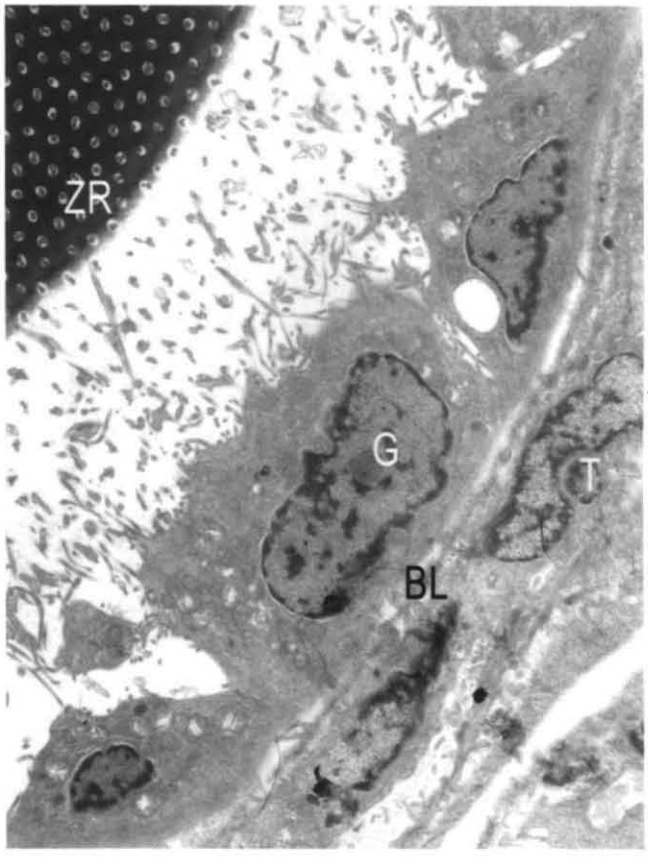



Plate 16. Electron micrograph of vitellogenic oocytes with well developed basal lamina . Note: zona radiata (ZR), basal lamina (BL), granulosa cell (G), thecal cell (T). (4000X).

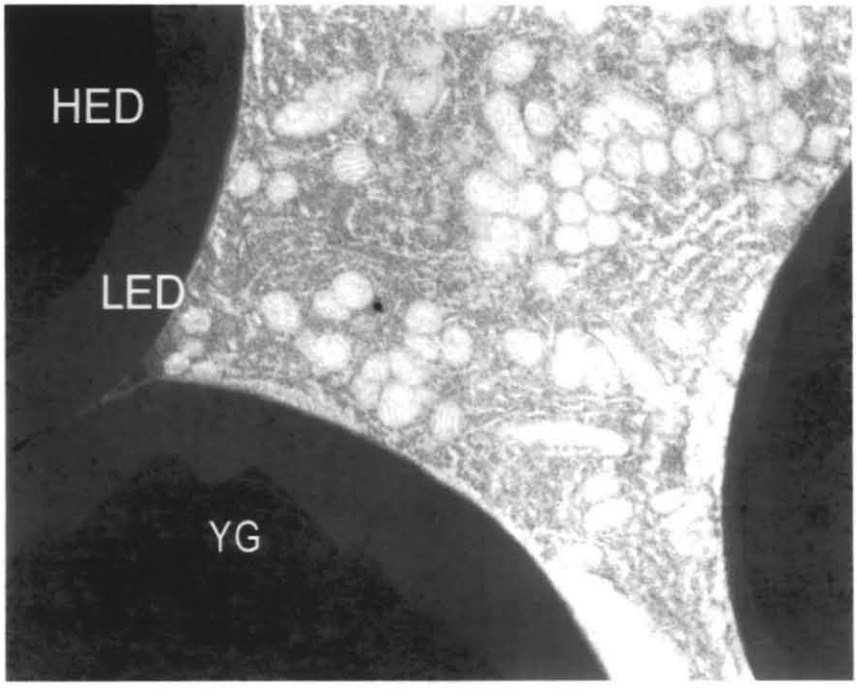



Plate 17. Ultra structure of protein yolk globule (YG). electron micrographs showing transitional yolkspheres. Note the central High Electron Dense (HED) layer and surrounded by Low Electron Dense (LED) fluid layer. (6000X)

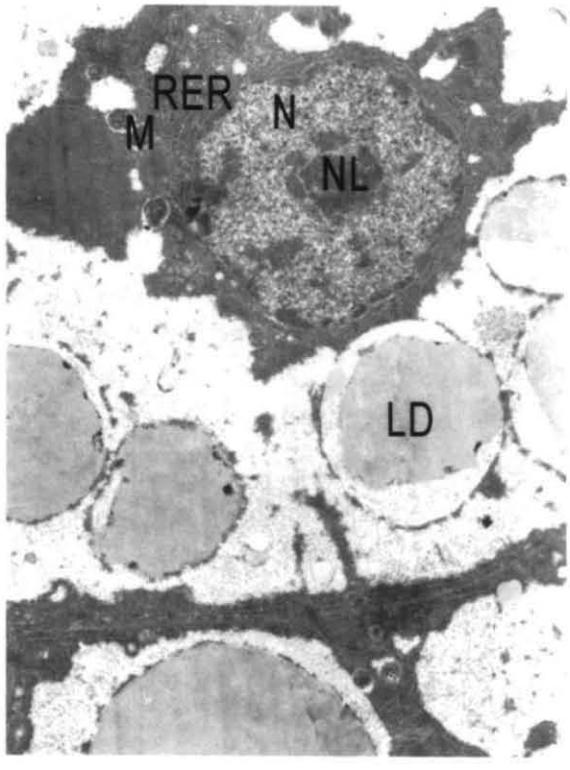



Plate 18. Hepatocyte of immature female E. diacanthus.
Note; nucleus (N), nucleolus (NL),
rough endoplasmic reticulum (rER), mitochondria (M).
(8000X).

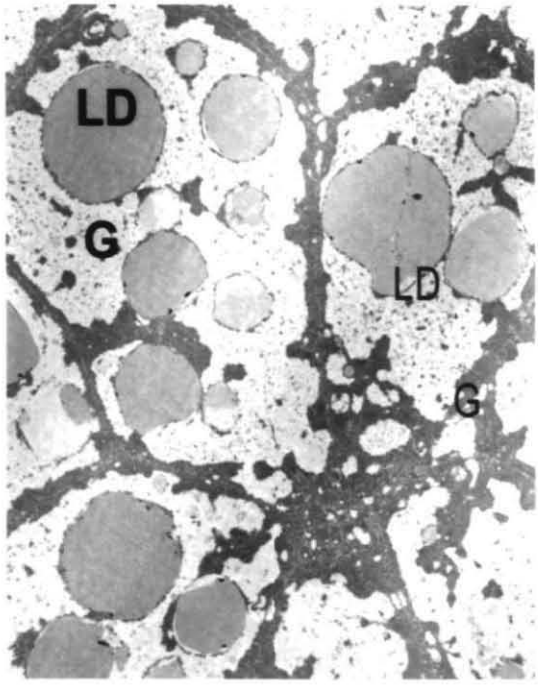



Plate 19. Hepatocyte of immature female *E. diacanthus*. with
lipid droplets (LD) and dispersed glycogen granules (G).
(4000X).

**Maturing**

Electron dense cytoplasm is seen in the maturing female hepatocytes of *E. diacanthus*. Lipid droplets are scarce in the cytoplasm compared to immature female hepatocytes. Glycogen granules are scattered in the cytoplasm (Plate.20)

**Ripe**

Hepatocytes of *E. diacanthus* are having dense rough endoplasmic reticulum with flat cisternae. Cytoplasm contains dense electron regions with few smooth endoplasmic reticulum. In the ripe female hepatocytes enlarged mitochondria are observed (Plate. 21, 22, and.23).

### 4.2.4 Hermaphroditism

In the present study *E. diacanthus* gonadal samples were collected onboard FSI cruises. Histological observations of the gonads have revealed the presence of transitional stage of gonads and protogynous mode of reproduction of the candidate species

Ovary in its early stages of transition into testis was observed. Moe(1969) described this stage as transitional stage. This stage is a temporary one. Ovary was observed with early stages of spermatogonial proliferation and degenerating oocytes .(Plate.24). In later stages of male germcells development with spermatocytes some regressing oocytes were noticed.(Plate. 25).

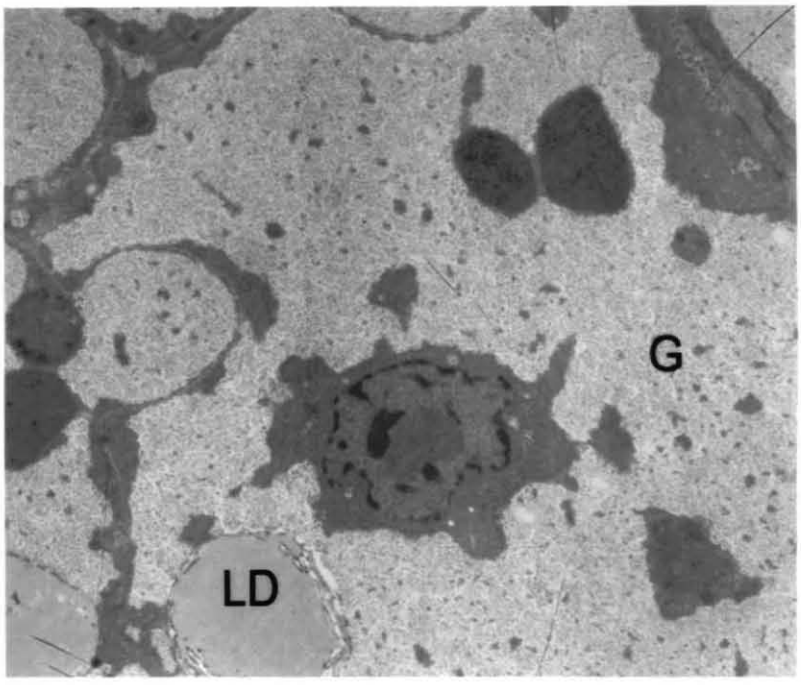



Plate 20. Hepatocyte of maturing female *E. diacanthus* with electron dense cytoplasm and scattered glycogen granules. (5000X)

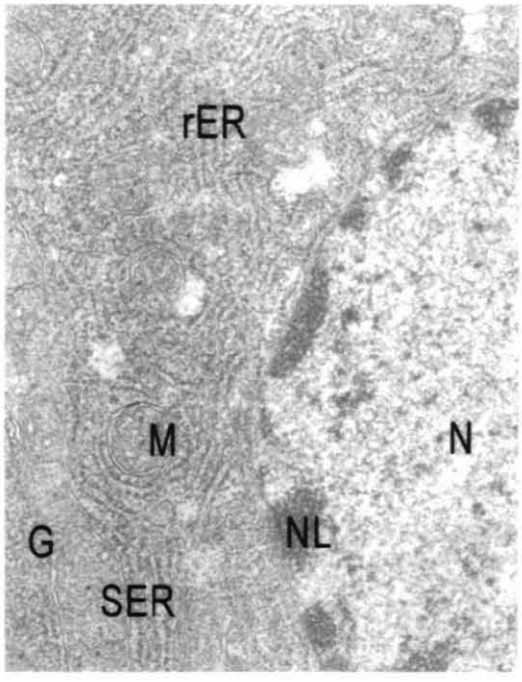



Plate 21. Electron micrograph of hepatocyte of female *E. diacanthus* in mature / ripe stage. Note: rough endoplasmic reticulum (rER) with parallel cisternae, smooth endoplasmic reticulum (SER),, glycogen granules (G). (30000x)

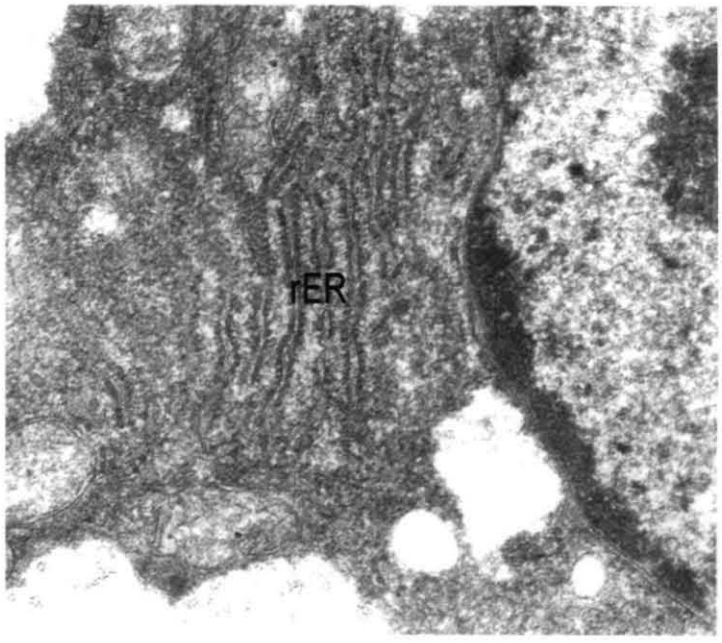



Plate 22. Electron micrograph of ripe female hepatocyte with dense rough endoplasmic reticulum (rER) with flat cisternae.(35000X)

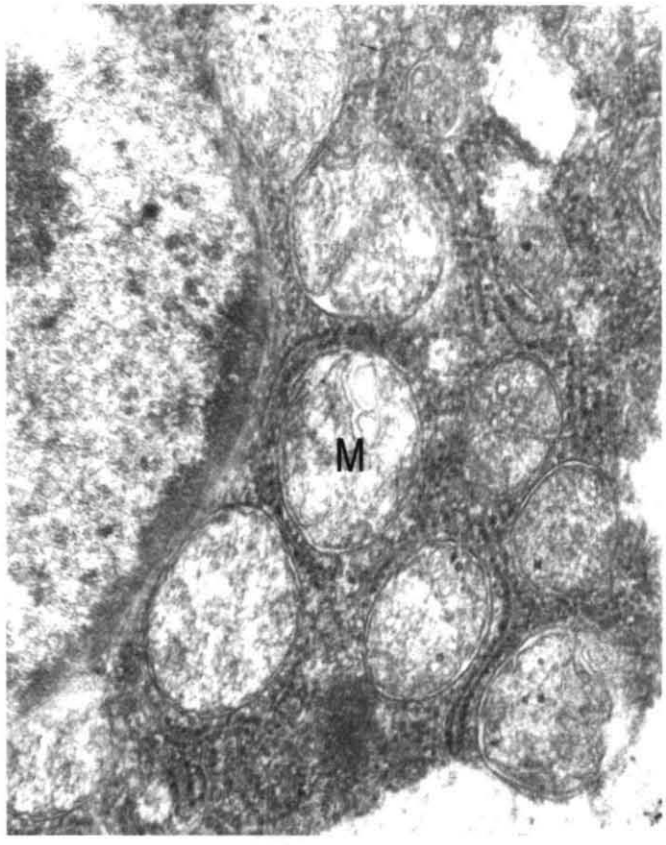



Plate 23. Electron micrograph of ripe female hepatocyte with dense and enlarged mitochondria (M). (35000X)

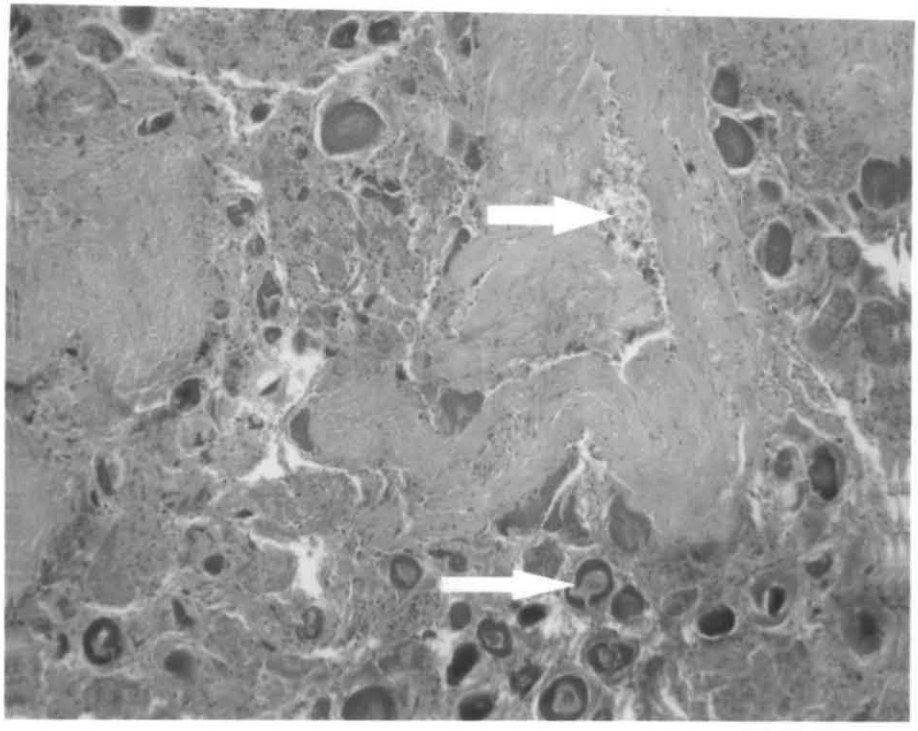


Plate 24. Light micrograph of transitional stage ovary of *E. diacanthus* with regressing oocytes and spermatogonia. Note: upper arrow indicates spermatogonia and lower arrow indicates regressing oocytes. (40X)

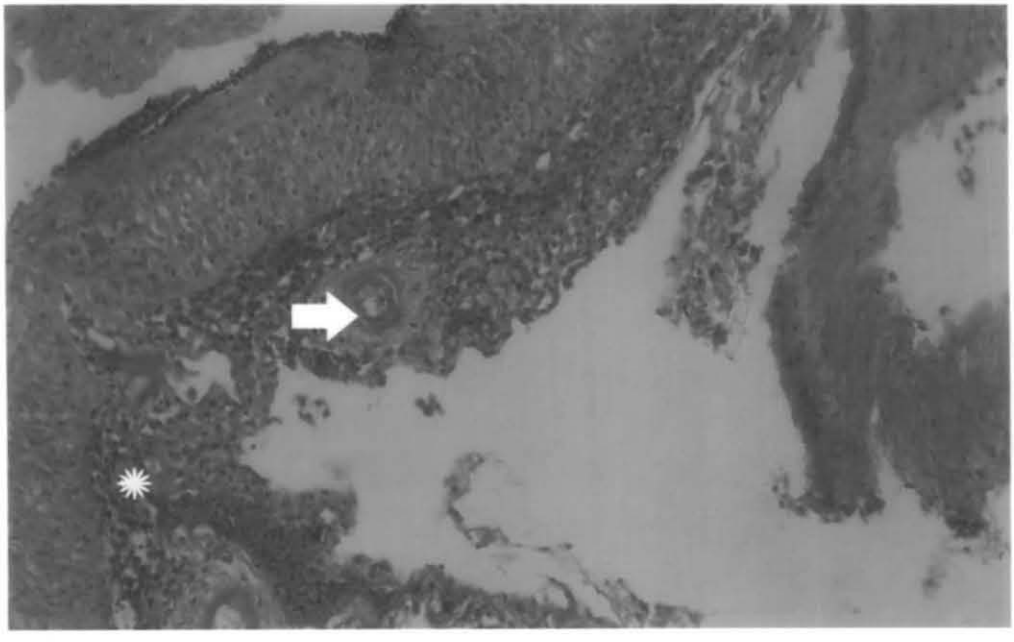


Plate 25. Light micrograph of E. diacanthus gonad with spermatocytes and regressing oocytes. Note: Arrow indicates regressing oocyte and asterisk indicates spermatocytes. (20X)

## 4.3 Electrophoresis

### 4.3.1. Characterisation of vitellogenin

Non denaturing polyacrylamide gel electrophoresis (Native PAGE) of gonadal tissue homogenate and serum samples of different maturity stages was conducted to resolve the level of expression of yolk protein during the maturation of gonads of female *E. diacanthus*.

Denaturing Sodium Dodecyl Sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the serum samples of different maturity stages to understand the number of subunits in the vitellogenin and to decipher the molecular weight of the subunits. Tissue homogenates were not taken into consideration due to the poor expression of protein bands in the native PAGE.

#### 4.3.1.1. Ovarian tissue homogenates

**General proteins**

Immature *E. diacanthus* ovarian homogenate electrophoresed sample has expressed 10 protein bands (Lane.No.10) (Plate.26). The protein bands in ovarian tissue homogenates of the maturing stage appeared more intense than in immature ovarian homogenate. A total of five protein bands appeared in the sample (lane No.9) from ovarian homogenates of the maturing stage *E. diacanthus*. Same number of protein bands (N = 4) were expressed in matured male (Lane. No. 8; $Rf_1$= 0.011, $Rf_2$ = 0.091, $Rf_3$ = 0.467, $Rf_4$ = 0.996) and female tissue homogenates (Lane. No.7; $Rf_1$ = 0.155, $Rf_2$ = 0.437, $Rf_3$ = 0.824, $Rf_4$ = 0.996). The characteristic protein bands obtained in the ripe female ovarian homogenate were not present in the male gonadal tissue homogenates. The protein band ($Rf_1$= 0.155) of ripe female ovarian homogenate is more prominent among all the protein bands as expressed in the native PAGE of tissue homogenates. (Table 7; Plate.26& 27);

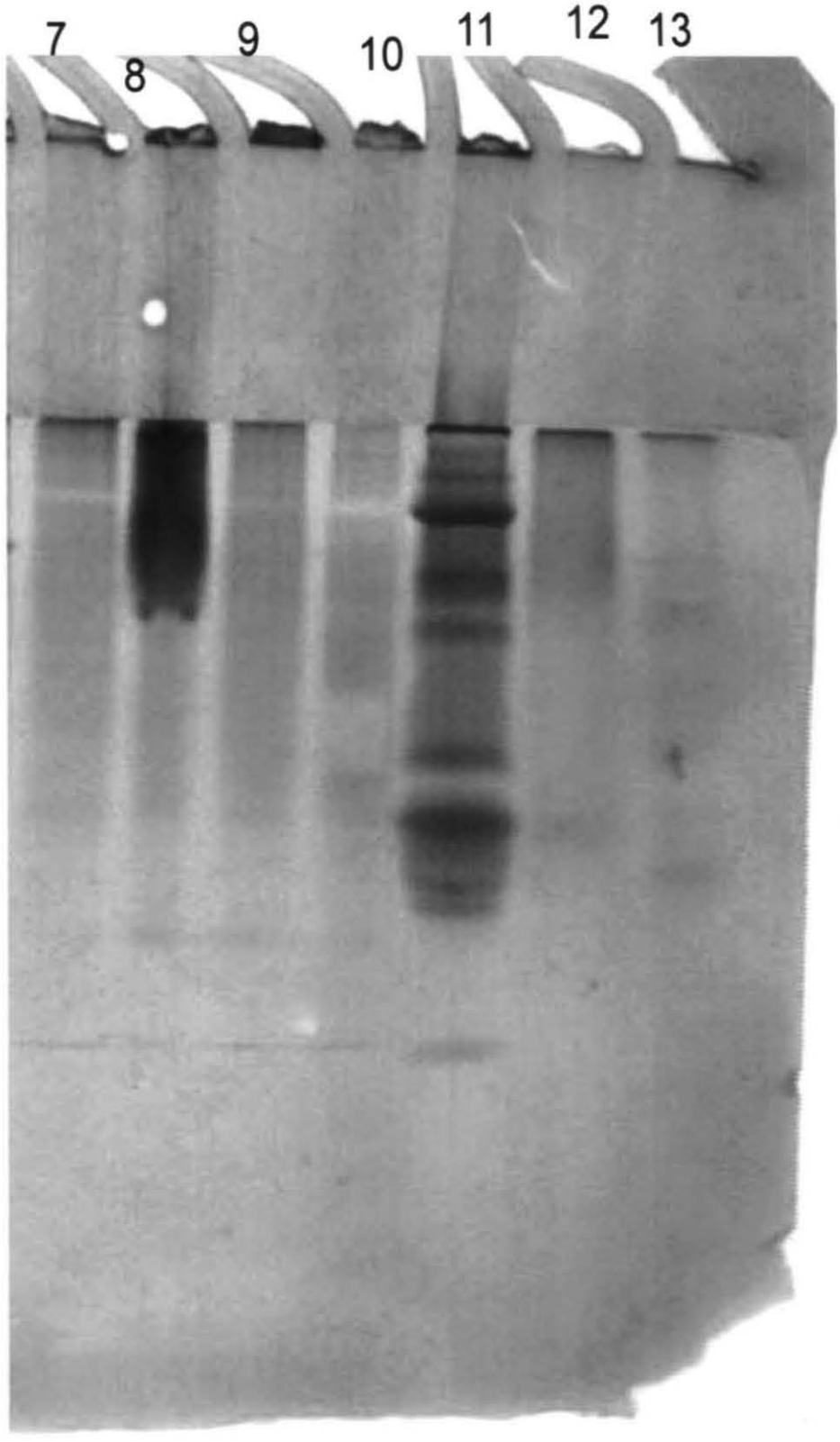



Plate 26. Native PAGE gel electrophoretic pattern of serum and ovarian homogenates. Lane.7- Mature male tissue homogenate Lane. 8- Mature/ Ripe female ovarian homogenate, Lane. 9- Maturing female ovarian homogenate, Lane. 10 - Immature female ovarian homogenate, Lane. 11- Mature/ Ripe female serum, Lane. 12- Maturing female serum, Lane. 13- Immature female serum

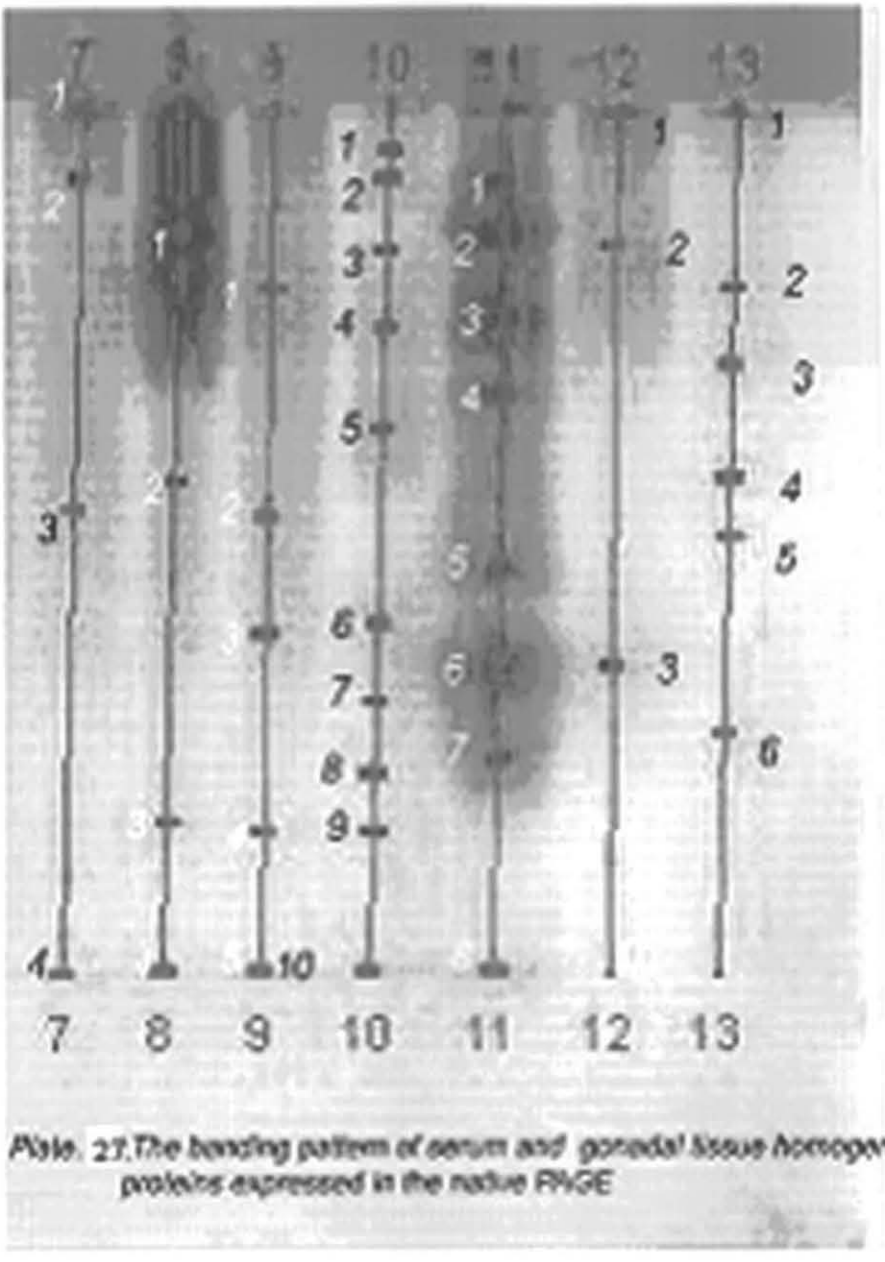



Plate. 27.The banding pattern of serum and gonadal tissue homogenate proteins expressed in the native PAGE

Table 7

Relative Fraction (Rf) values of tissue homogenate and serum proteins of various maturity stages expressed in the native PAGE

| Lane number | Band number | Rf - value |
|---|---|---|
| 7 | 1 | 0.011 |
| 7 | 2 | 0.091 |
| 7 | 3 | 0.467 |
| 7 | 4 | 0.996 |
| 8 | 1 | 0.155 |
| 8 | 2 | 0.437 |
| 8 | 3 | 0.824 |
| 8 | 4 | 0.996 |
| 9 | 1 | 0.213 |
| 9 | 2 | 0.472 |
| 9 | 3 | 0.606 |
| 9 | 4 | 0.833 |
| 9 | 5 | 0.996 |
| 10 | 1 | 0.053 |
| 10 | 2 | 0.085 |
| 10 | 3 | 0.17 |
| 10 | 4 | 0.258 |
| 10 | 5 | 0.371 |
| 10 | 6 | 0.594 |
| 10 | 7 | 0.682 |
| 10 | 8 | 0.763 |
| 10 | 9 | 0.83 |
| 10 | 10 | 0.993 |
| 11 | 1 | 0.085 |
| 11 | 2 | 0.149 |
| 11 | 3 | 0.245 |
| 11 | 4 | 0.33 |
| 11 | 5 | 0.528 |
| 11 | 6 | 0.645 |
| 11 | 7 | 0.748 |
| 11 | 8 | 0.993 |
| 12 | 1 | 0.011 |
| 12 | 2 | 0.16 |
| 12 | 3 | 0.638 |
| 13 | 1 | 0.011 |
| 13 | 2 | 0.21 |
| 13 | 3 | 0.299 |
| 13 | 4 | 0.427 |
| 13 | 5 | 0.495 |
| 13 | 6 | 0.719 |

#### 4.3.1.2 Glycolipoproteins with Calcium moiety

In the present study *E. diacanthus* ripe female ovarian homogenate protein bands as expressed in the native PAGE exhibited the positive staining with special stains Periodic acid Schiff's reagent (Plate. 28), Sudan Black (Plate. 29) and Alizarin red (Plate. 30) for glycoconjugate, lipoconjugate and calcium moiety respectively.

In the immature and maturing stages of the female and male tissue homogenates, the bands are not as prominent as observed in ripe female tissue homogenate.

### 4.3.1.3 Female specific proteins

The protein bands 5, 6 and 7 (Lane No. 11; $Rf_5$ = 0.528, $Rf_6$ = 0.645, $Rf_7$ = 0.748) of the ripe female serum samples are very specific to maturity stage and gender. These bands were not seen in other maturity stages as well as in male tissue homogenates. The protein band ($Rf_1$ =.0155) in ripe female appeared to be more distinct as compared to those of male and female tissue homogenates of other stages. (Plate.27, Table-7)

## 4.3.2. Blood serum

**General proteins**

As evident from native PAGE electrophoretic pattern six protein bands were expressed in the immature serum sample of *E. diacanthus* (Lane. No.13). Maturing stage female serum has showed only three bands (Lane. No.12) where as blood serum obtained from the ripe female *E. diacanthus* has expressed eight protein bands with high intensity (Lane No. 11; $Rf_1$ = 0.085, $Rf_2$ = 0.149, $Rf_3$ = 0.245, $Rf_4$ = 0.33, $Rf_5$ = 0.528, $Rf_6$ = 0.645, $Rf_7$ = 0.748, $Rf_8$ = 0.993). The protein bands appeared in the ripe female serum samples was not obvious in the immature and maturing female serum samples. (Table -7, Plate.27):

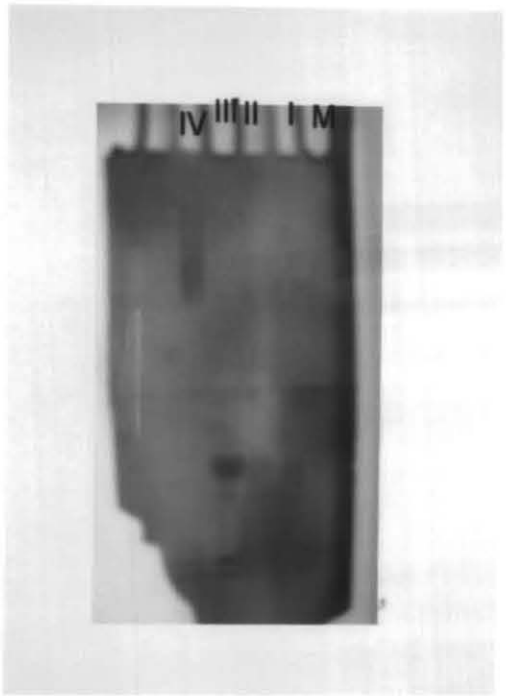



Plate 28. Native PAGE of yolk protein stained with PAS
I- Immature II- Maturing III- Ripe serum samples IV- Ripe ovarian homogenate M- Male gonadal tissue homogenate

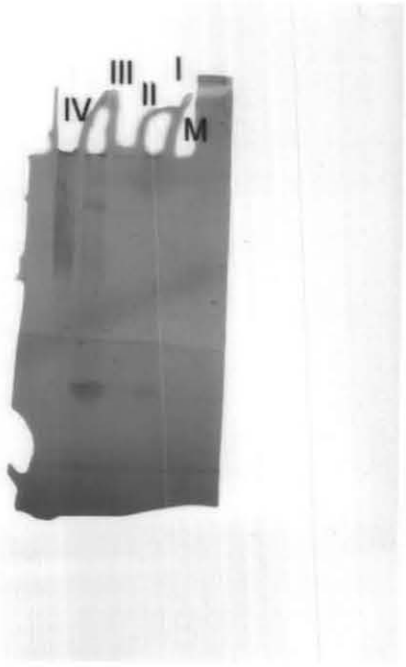



Plate 30. Native PAGE of serum and ripe ovarian homogenate proteins stained with Alizarin red. M- Male gonadal tissue
I- Immature II- Maturing III- Ripe serum
IV- Mature female ovarian homogenate

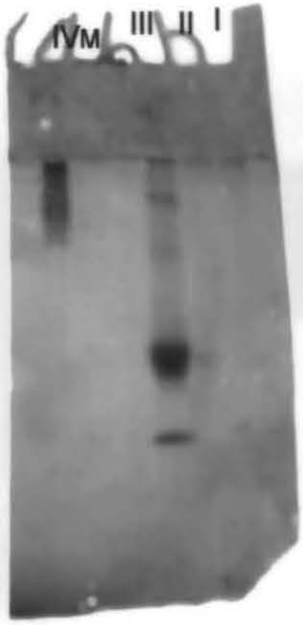



Plate 29. Native PAGE of yolk protein stained with Sudan Black B. I- Immature, II- Maturing III- Mature /Ripe serum, IV- Ripe female ovarian homogenate, M- Male gonadal tissue homogenate

### 4.3.2.1 Glycolipoprotein with calcium moiety

The ripe female serum proteins have shown positive lipoprotein staining with Sudan Black B. A high molecular weight protein fraction that has shown positive reaction to Sudan Black B, but was absent in Periodic acid Schiff's reagent and Alizarin red staining. In *E. diacanthus* ripe female serum 5, 6 and 7 protein bands ($Rf_5$ = 0.528, $Rf_6$ = 0.645, $Rf_7$ = 0.748) expressed in the native PAGE are prominent and characteristic with this stage and are absent in other stages. Among these high molecular protein bands 6 and 7 protein bands expressed positive staining with Periodic acid Schiff's reagent, Sudan Black B and Alizarin red (Plate. 28, 29 and 30). These special stained bands were not observed in the immature and maturing serum samples and are evident in ripe female serum sample.

### 4.3.2. 2 Molecular weight estimation

The serum proteins obtained from ripe female samples of *E. diacanthus* expressed four distinct protein bands as appeared in SDS-PAGE electrophoretic pattern (Plate. 31). Three distinct protein bands of high molecular weight ( $Rf_5$ = 0.528, $Rf_6$ = 0.645, $Rf_7$ = 0.748) were evident in native PAGE for ripe female *E. diacanthus*.(Table 7). Three prominent protein bands having molecular weight (MW) of 214.80kDa, 407.5kDa and 583.59kDa (Lane No. 11) have been observed in serum samples from ripe female *E. diacanthus*. In the maturing stage the aforesaid bands are not apparent as appeared from the native gel electrophoresis (Lane No. 12). As evident from the ripe SDS gel electrophoretic pattern four prominent bands at 214.8kDa, 262.6kDa, 321.0kDa, and 407.5kDa are apparent which are not present in maturing sample (Plate. 31). One extra band appeared in ripe serum sample as noticed in SDS-PAGE where as only three bands were observed in the native PAGE electrophoretic pattern.

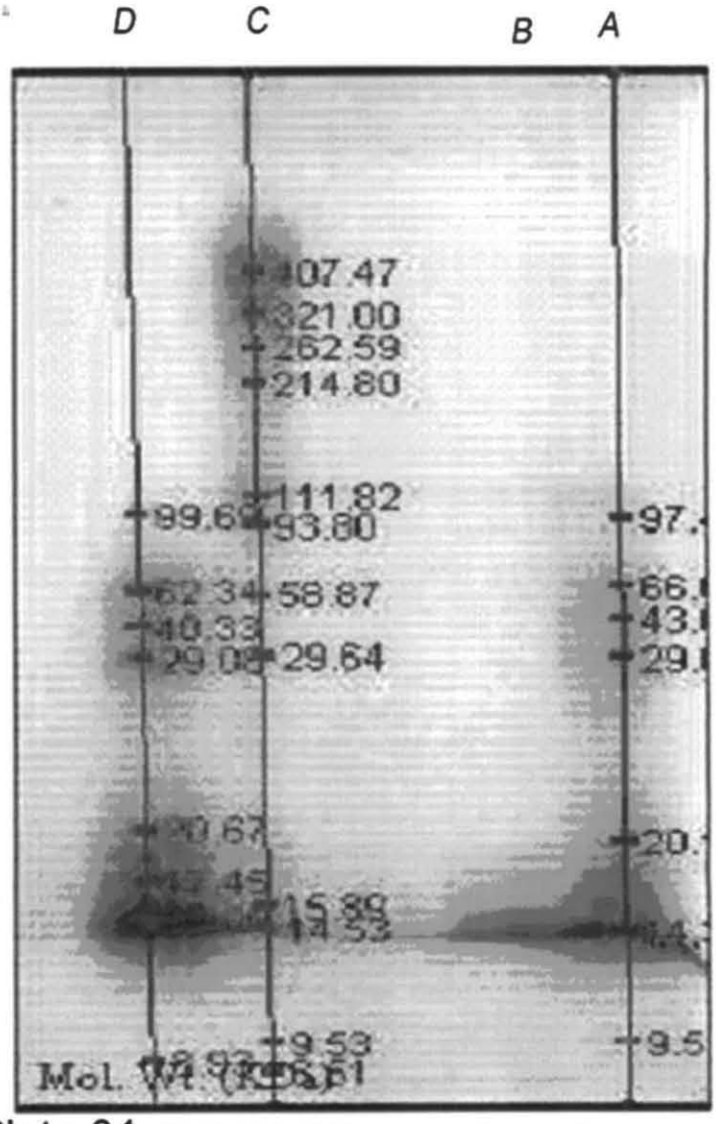



Plate 31. *SDS-PAGE Serum protein gel electrophoretic profile of various maturity stages .*

*A- Medum range Marker B- Immature stage*

*C- Ripe stage D- Maturing stage*

*Note: Molecular weights of respective protein bands Kilodalton*

## 4.4 Biochemical changes during maturation

In the present study, the biochemical composition of four tissues viz., muscle, liver, ovary and blood serum of *Epinephelus diacanthus* has been studied and the results are detailed below. Seven major parameters such as the moisture, proteins, lipids, carbohydrates, cholesterol, carotenoid and ash have been estimated in relation to the maturation stages.

## Proximate composition analysis

### 4. 4. 1. Muscle

**Total Moisture**

Highest moisture percentage was observed in the maturity stage I (76.84%), and the moisture percentage gradually reduced in the stage II and Stage III, showing an inverse relation (Table - 8, Fig. 7)

**Total Protein**

Trend in total protein percentage of muscle showed a positive relation. Protein percentage increased from stage I (18.8) to Stage III (32.8).

**Total Carbohydrates**

Carbohydrate percentage in the muscle tissue during the maturation varied between 1.3 (stage III) to 3.09 (stage I). Carbohydrate percentage was high in maturity state I and the maturity stages II and III showed slight variation.

**Total Lipids**

Lipids content in the muscle was 2.468 % in stage I and it increased slightly to 2.72 % in stage II. Even though the lipid content of muscle increased from stage I to stage II of gonadal maturation, it declined to 1.648% in the stage III.

**Table 8**

**Biochemical constituents content variation in the muscle In relation to the maturation of gonads of the grouper, *E. diacanthus* ( Mean ± SD)**

| Stage | Moisture (%) | Total Proteins (%) | Total Carbohydrates(%) | Total lipids (%) | Total Cholesterol(%) | Total Carotenoids(µg/g) | Ash(%) |
|---|---|---|---|---|---|---|---|
| I | 76.84 ± 2.74 | 18.80 ± 0.049 | 3.096 ± 0.009 | 2.46 ± 0.012 | 1.13 ± 0.016 | 3.8 ± 0.037 | 1.81 ± 0.007 |
| II | 72.15 ± 2.03 | 24.64 ± 0.018 | 1.372 ± 0.013 | 2.72 ± 0.022 | 0.83 ± 0.023 | 5.19 ± 0.015 | 0.55 ± 0.002 |
| III | 68.22 ± 1.55 | 32.81 ± 2.107 | 1.723 ± 0.029 | 1.648±0.019 | 0.762 ± 0.015 | 8.71 ± 0.016 | 0.39 ± 0.001 |

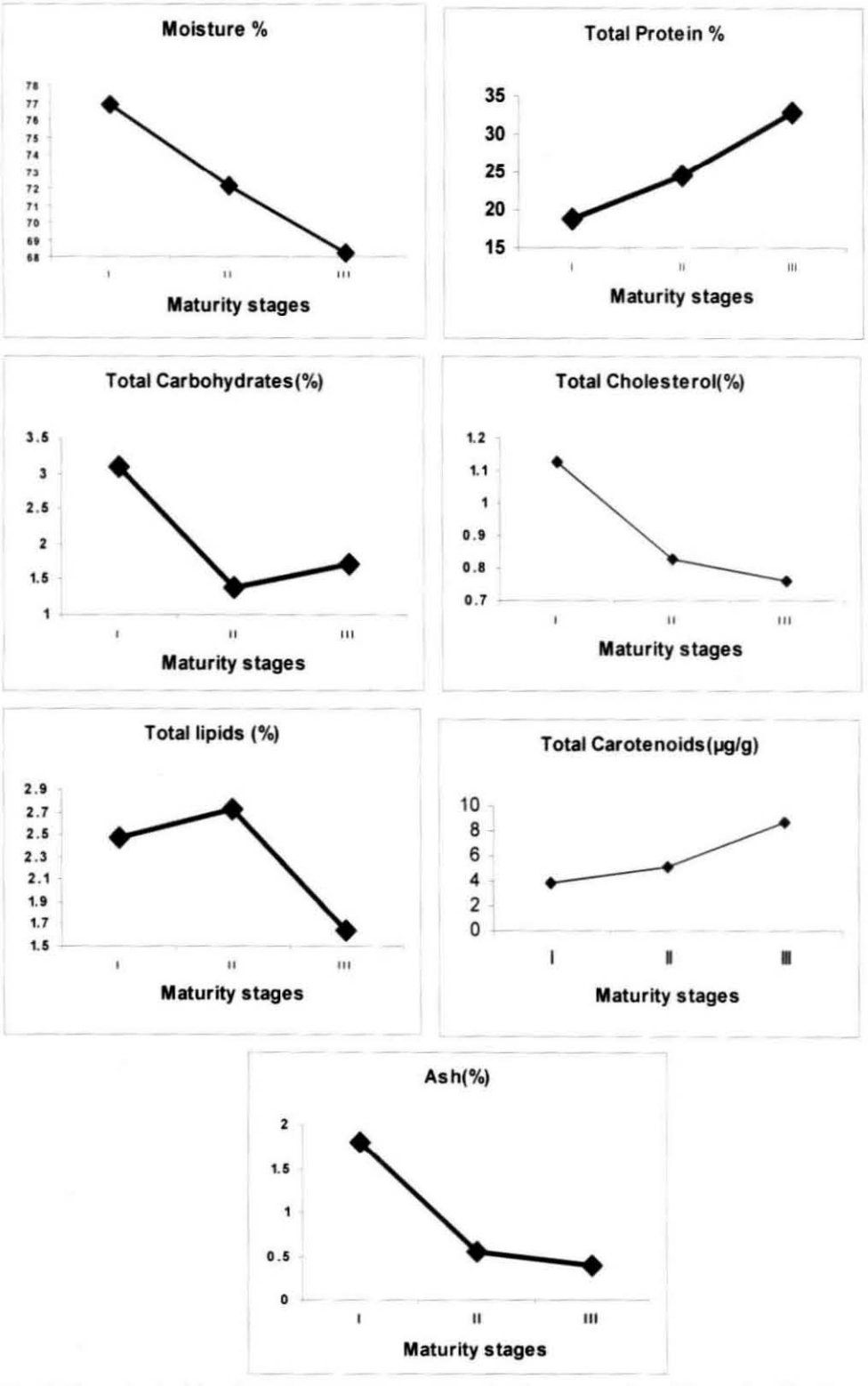



Fig.7 Trends in biochemical composition in the muscle of female *E. diacanthus* during ovarian development

**Total Cholesterol**

Muscle cholesterol percentage decreased from stage I (1.13%) to stage III (0.762%), showing an inverse relationship with the maturation of gonad.

**Total Carotenoids**

Total carotenoids percentage in the muscle tissue showed a positive relationship with maturity stages. In stage I it showed the value of 3.8μg/g and in the stage III it was 8.71μg/g.

**Ash %**

Muscle ash percentage showed an inverse relationship. Ash percentage showed the decreasing trend from stage I (1.81%) to stage III (0.39%).

## 4. 4. 2. Liver

**Total Moisture**

In the liver, moisture percentage showed an inverse relation. The moisture percentage decreased from 66.0 in stage I to 57.2 stage III. (Table - 9, Fig.8)

**Total Protein**

Protein percentage showed a positive relationship. Highest protein percentage was observed in the stage III (22.30%) and the lowest in stage I ( 11.5)

**Total Carbohydrates**

Liver carbohydrate percentage showed an increase and decrease with gonadal stage progression. In stage I it showed 13.23% and it decreased in stage II (10.96 %) and increased in stage III (14.27%).

**Table 9**

**Biochemical constituent contents variation in the liver in relation to the maturation of gonads in the female grouper, *E. diacanthus* (Mean ± SD)**

| Stage | Moisture (%) | Total Protein(%) | Total Carbohydrates (%) | Total lipids(%) | Total Cholesterol(%) | Total Carotenoids (µg/g)) | Ash(%) |
|---|---|---|---|---|---|---|---|
| I | 66.02 ±0.90 | 11.59± 0.255 | 13.23 ± 0.025 | 16.17± 0.09 | 3.42 ± 0.16 | 3.6 ± 0.07 | 0.81 ± 0.005 |
| II | 61.60 ±0.78 | 15.029±0.094 | 10.96 ± 0.102 | 11.66 ± 0.24 | 2.27 ± 0.14 | 7.14 ± 0.054 | 0.55 ± 0.002 |
| III | 57.20 ± 0.56 | 22.30 ±0.468 | 14.27 ± 0.046 | 7.08 ± 0.17 | 1.67 ± 0.02 | 11.3 ± 0.16 | 0.39 ± 0.003 |

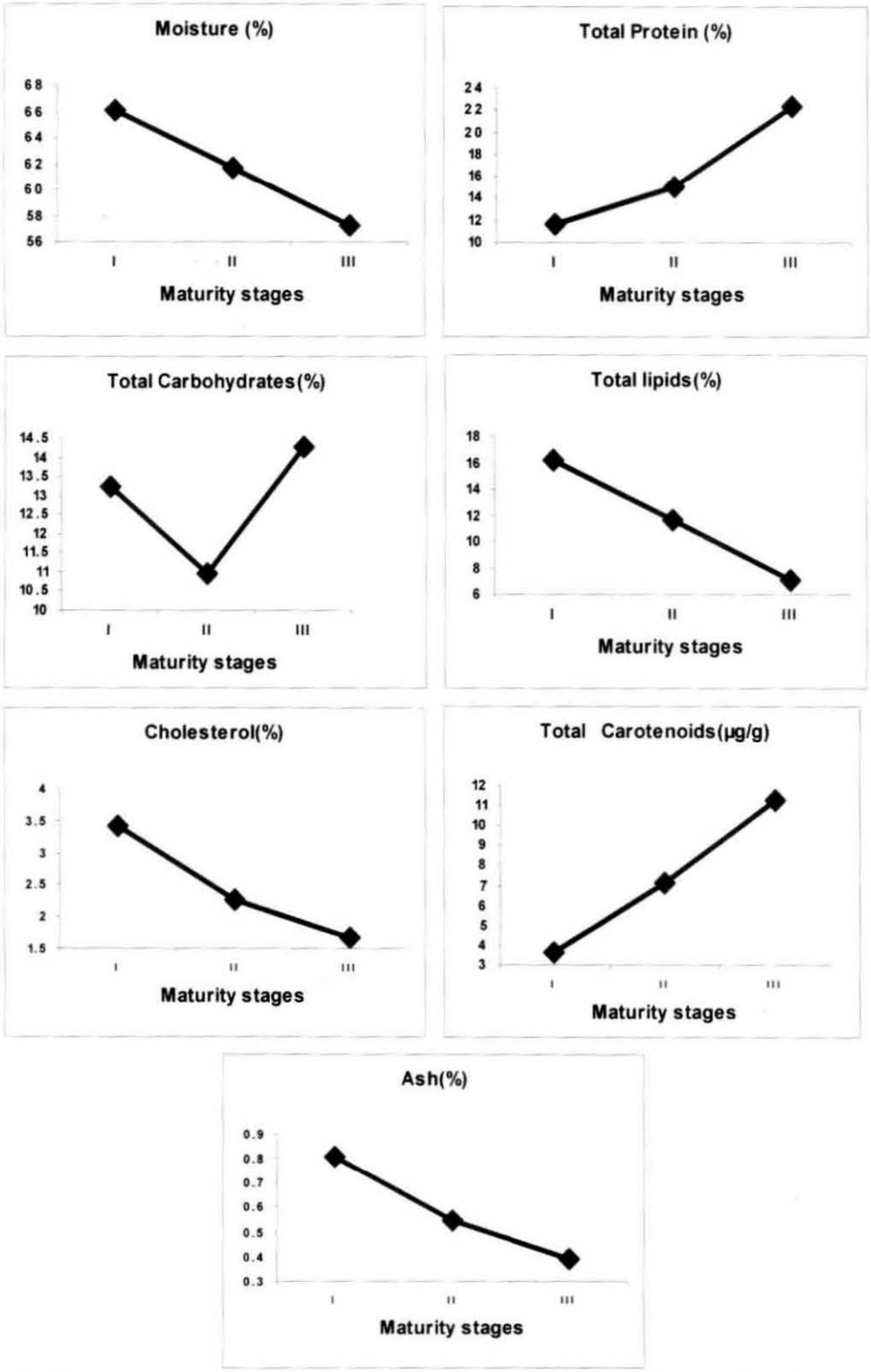



Fig.8 Trends in biochemical composition in the liver of female *E. diacanthus* during ovarian development.

**Total lipids**

Lipid percentage of the liver showed an inverse relationship. In stage I the value was 16.17% and it decreased to 7.08 % in stage III.

**Total cholesterol**

The total cholesterol percentage in liver tissue was higher than any other body tissues. Cholesterol percentage showed an inverse relationship, decreasing from stage I (3.42) to stage III(1.67)

**Total Carotenoids**

The carotenoids content of liver showed a steady increase from stage I to stage III showing a positive relationship. Highest carotenoids content was observed in stage III (11.3μg/g) and lowest in stage I (3.6μg/g). Highest variations in the carotenoids content of different fishes of same stage was observed in stage III (SD = ± 0.16).

**Ash**

The percentage of ash showed an inverse relationship. Highest ash percentage was observed in stage I (0.81%).and the lowest ash percentage was in stage III (0.39%).

## 4.4. 3. Blood serum

**Total protein**

Blood serum total protein percentage showed a positive trend. In stage I the value was 6.52 and in stage III it was 10.98. (Table – 10 and11; Fig. 9)

**Table 10**

**Biochemical constituents content variations in the blood serum in relation with the maturation of gonads in the female grouper, *E. diacanthus*(Mean ± SD)**

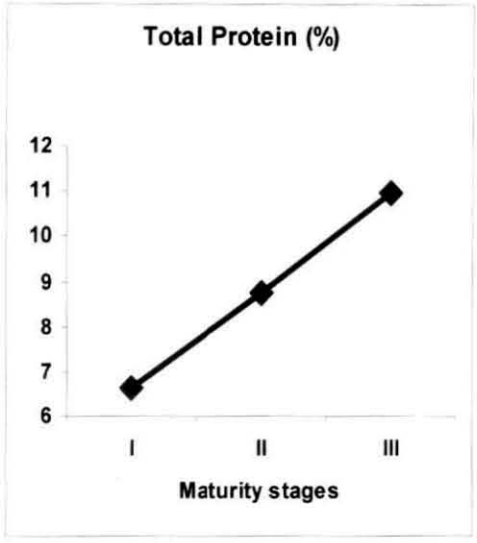
| Stage | Total Protein (%) | Total Carbohydrates (%) | Total lipids (%) | Total Cholesterol(%) | Total Carotenoids(µg/ml) |
|---|---|---|---|---|---|
| I | 6.61 ± 0.013 | 6.52 ± 0.02 | 1.86 ± 0.039 | 0.45 ± 0.019 | 0.17 ± 0.01 |
| II | 8.73 ± 0.001 | 8.27 ± 0.07 | 2.13 ± 0.016 | 0.37 ± 0.006 | 0.33 ± 0.02 |
| III | 10.98 ± 0.05 | 6.83 ± 0.11 | 2.61 ± 0.05 | 0.63 ± 0.018 | 0.95 ± 0.01 |

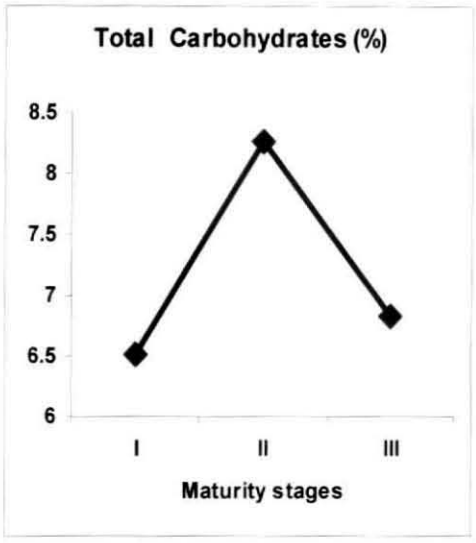


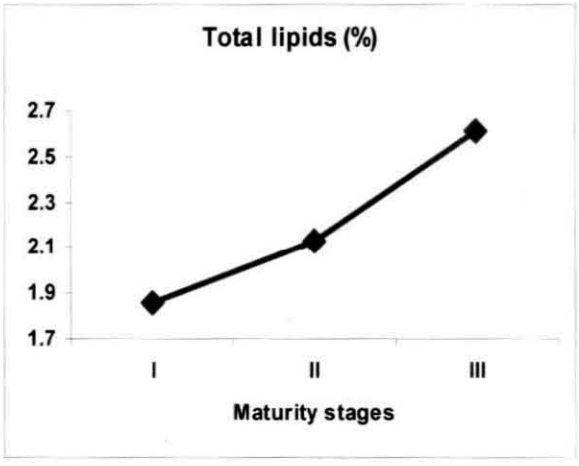
**Table 11**

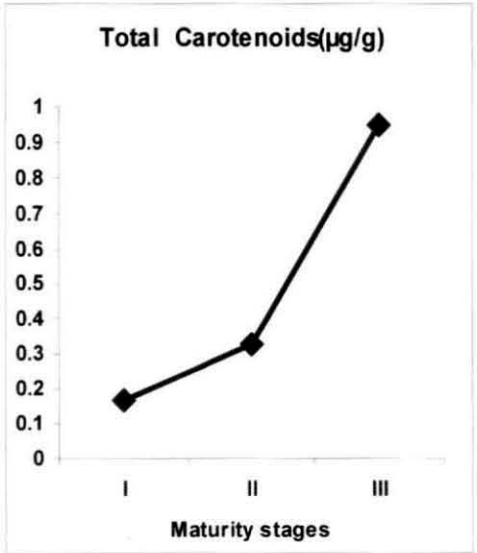
**Statistical significance test results for serum biochemical parameters**

| Parameter | TOTAL proteins | TOTAL Carbohydrates | Total lipids | Total cholesterol | Total carotenoids |
|---|---|---|---|---|---|
| F- calculated value | 20143.76 | 722.150 | 1812.424 | 695.353 | 2066.065 |
| Remarks | p<0.01 | p<0.01 | p<0.01 | p<0.01 | p<.0.01 |



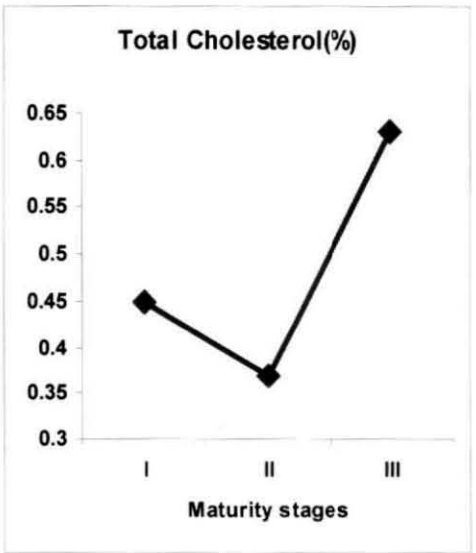




Fig.9 Trends in biochemical composition in the blood serum of female *E. diacanthus* during ovarian development.

**Total Carbohydrates**

Serum carbohydrates percentage showed a rise and fall trend. The carbohydrates percentage in stage I was 6.52 and it increased in stage II to 8.29% and again it fell to 6.83% in stage III

**Total lipids**

Blood serum lipids showed a positive relation. There was a steady increase in values from stage I to stage III. Highest total lipid percentage was observed in stage III (2.61%). In stage I (1.86%) lowest lipid percentage was observed.

**Total Cholesterol**

Serum cholesterol levels showed rise and fall trend with the maturation of gonads .In stage I the value was 0.45 and the value was fell to 0.37 in stage II again it increased to 0.63 in stage III.

**Total carotenoids**

Blood carotenoids levels are low compared to liver, body and ovary. Carotenoid percentage showed a positive relationship with the increasing maturity stages. In stage I the value was 0.17μg/g and it rose to 0.95μg/g in stage III.

## 4.4.4 . Ovary

**Moisture**

Ovarian moisture percentage showed inverse relationship showing a steady decrease from stage I (71.66 %) to stage III(60.58 %).(Table - 12, Fig. 10)

**Table 12**

**Biochemical constituents content variations in the ovary in relation to the maturation in the female grouper, *E. diacanthus* (Mean ± SD)**

| Stage | Moisture (%) | Total Proteins (%) | Total Carbohydrates (%) | Total lipids (%) | Total Cholesterol (%) | Total Carotenoids (µg/g) | Ash(%) |
|---|---|---|---|---|---|---|---|
| I | 71.66 ± 1.15 | 14.02± 0.059 | 1.46 ± 0.008 | 6.56 ± 0.01 | 2.06 ± 0.03 | 1.42 ± 0.03 | 0.820 ± 0.005 |
| II | 63.56 ± 0.25 | 17.93 ± 0.07 | 1.23 ± 0.001 | 7.89 ± 0.06 | 1.65 ± 0.019 | 2.69 ± 0.05 | 1.01 ± 0.008 |
| III | 60.55 ± 0.19 | 24.81 ± 0.28 | 1.38 ± 0.005 | 13.48 ±0.04 | 1.27 ± 0.03 | 7.98 ± 0.021 | 1.20 ± 0.003 |

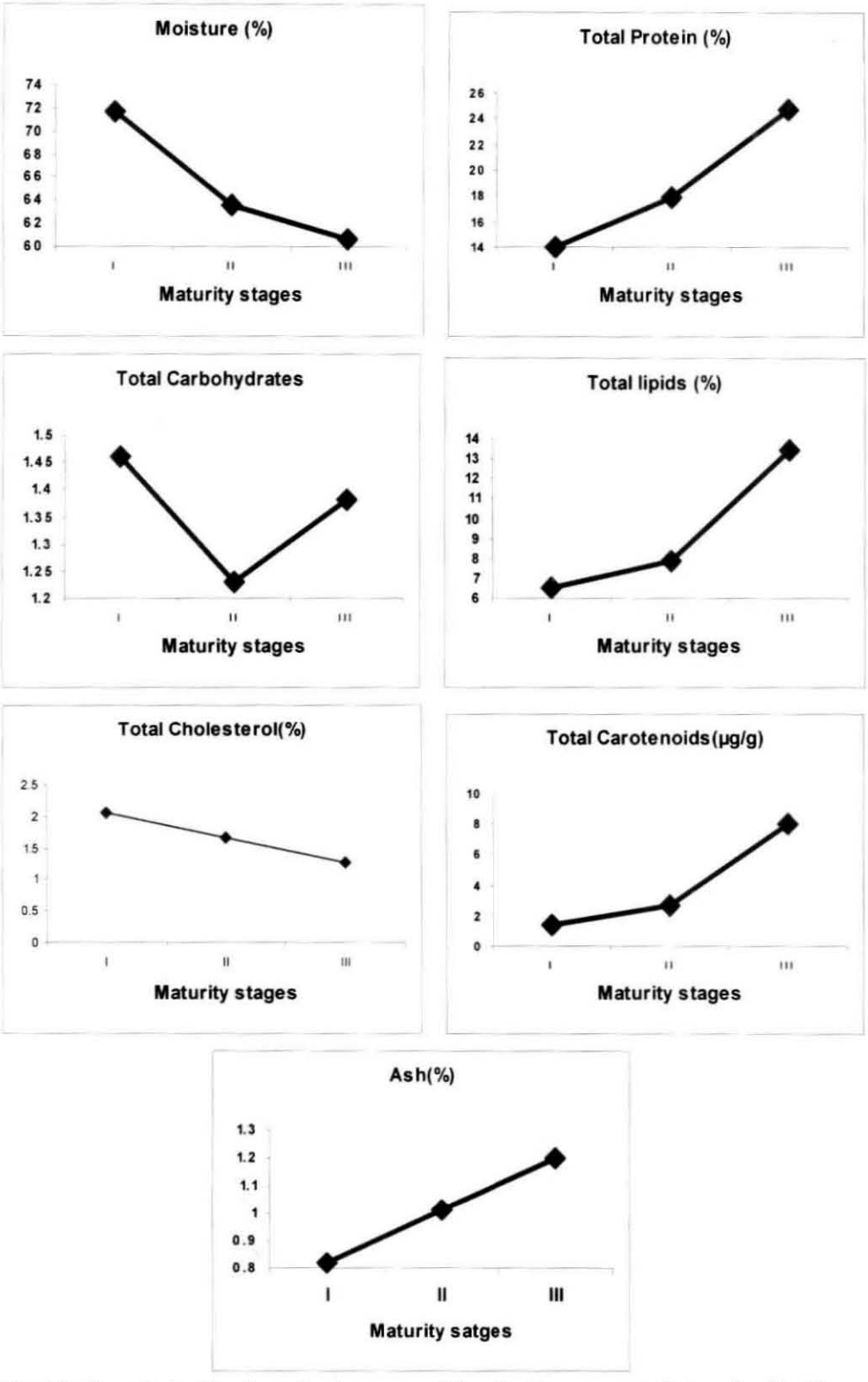



Fig.10 Trends in biochemical composition in the ovary of female *E. diacanthus* during ovarian development

**Total *proteins***

The total protein percentage in the ovary was observed having a positive relation with the maturation showing an increasing trend. In stage I the value was 14.02 % whereas in stage III it was 24.81%

**Total carbohydrates**

: The total carbohydrate content showed a fluctuating trend. High value was observed in stage I (1.43%); showed a downward trend in stage II (1.23 %); the value again rose in stage III (2.38 %).

**Total lipids**

Total lipid percentage showed a positive relationship by a gradual increase from stage I (6.56 %) to stage III.(13.48 %)

**Total Cholesterol**

Total cholesterol levels of ovary showed an inverse relationship showing a steady decline from stage I (2.06) to stage III(1.27%)

**Total Carotenoids**

Carotenoids levels in the ovary showed a positive relationship. In stage I the value was 1.42% and in the stage III it was 7.98%.

**Ash**

Ovary tissue contained more ash percentage than the liver. It showed a positive relation. In stage I, 0.82 % and in the Stage III it was 1.20 %.

### 4.4.5 Statistical Analysis

The data on biochemical analysis of all parameters of liver, ovary and body tissue for different maturity stages were analyzed by one way ANOVA using the SYSTAT version 7.0.1 and the significance was tested at 1% level.

The analysis of variance was worked out for each biochemical parameter in muscle liver and ovary to test significant changes (i) between different tissues at various stages of maturity (ii) between different stages of maturity in various tissues.

The results (Table - 13) indicated significant variance of all parameters between tissues and between stages at 1% level. Interaction between the two sources of variation (tissues and stages) was significant at 1% level.

The variation in the composition of blood serum (Table - 11) at different stages of maturity was investigated by one way ANOVA, applied separately for each biochemical component. The results showed significant variance of all parameters ($p < 0.01$) between different maturity stages.

**Table- 13**

**Statistical analysis and significance level of different parameters**

| Parameter | Source of variation | Calculated F value | Remarks |
|---|---|---|---|
| Moisture | Between tissues | 450.153 | p< 0.01 |
| | Between stages | 342.213 | p< 0.01 |
| | Interactions | 5.471. | p< 0.05 |
| Proteins | Between tissues | 7502.304 | p< 0.01 |
| | Between stages | 12192.519 | p< 0.01 |
| | Interactions | 106.394 | p< 0.01 |
| Carbohydrates | Between tissues | 5323.681 | p< 0.01 |
| | Between stages | 364947.823 | p< 0.01 |
| | Interactions | 2798.648 | p< 0.01 |
| Lipids | Between tissues | 29038.874 | p< 0.01 |
| | Between stages | 405.653 | p< 0.01 |
| | Interactions | 6783. 994 | p< 0.01 |
| Cholesterol | Between tissues | 18599.743 | p< 0.01 |
| | Between stages | 7581.936 | p< 0.01 |
| | Interactions | 1344.708 | p< 0.01 |
| Carotenoids | Between tissues | 9313.428 | p< 0.01 |
| | Between stages | 34038.98 | p< 0.01 |
| | Interactions | 884.534 | p< 0.01 |
| Ash | Between tissues | 32383.896 | p< 0.01 |
| | Between stages | 1325.083 | p< 0.01 |
| | Interactions | 2899.396 | p< 0.01 |

Note: p<0.01 - At 1% level of significance
p<0.05 - At 5% level of significance

# *Discussion*

# 5. DISCUSSION

## 5.1 Reproductive biology

Reproduction involves changes in oocytes growth and development during the process of gonadal maturation. With the advancement of maturation, oocytes accumulate energy reserves and enlarge for further need for the onset of embryogenesis. In the present study, *E. diacanthus* oocyte size increased from stage I to stage III of gonadal maturation. Yashiro *et al.* (1993) have observed the oocyte size increase with the progression of gonadal maturation in *E. malabaricus*. They have reported that the oocyte size increased from 0.28mm to 0.41mm with the advancement of vitellogenesis. The above results are similar with the observations made in the present study on *E. diacanthus*. Yeh *et al.* (2003) by working on *Epinephelus tukula* had noticed the oocyte diameter increase from immature stage (120µm) to ripe stage (552µm), which is very similar to *E. diacanthus*. The egg diameter of *E. morio* was found to be less than1mm by Moe (1969). Thompson and Munro (1978) have found that in *Epinephelus guttatus* with the maturation of gonads the egg diameter varies between 0.70mm and 0.90mm. Brule and Deniel (1996) also observed the similar trend in oocyte cyclic development in the immature oocyte (54µm) to ripe oocytes (897µm) in red grouper, *E. morio*. In the present study, largest oocyte diameter was 650µm in ripe stage ovary of *E. diacanthus*. Tessy (1994) also has observed the largest oocyte diameter as 600µm in *E. diacanthus*. Powell and Tucker (1992) reported eggs of 0.92mm diameter in *E. striatus*.

Fecundity information of a species is essential for estimating seed propagation capacity and spawning population of the species concerned. Fecundity of the individual fish is determined from the total number of mature ova that are destined to be shed at the ensuing spawning season. In the present study *E. diacanthus* gonad weight in relation to the total fecundity showed linear relationship. The relation has showed significance ($r^2$ = 0.5841). Tessy (1994)

has also observed similar observation between gonad weight and fecundity in *E. diacanthus* and *E. bleekeri*. Bouain and Siau (1983) have reported that the fecundity is very closely related to the weight of the gonads in *E. aeneus*.

The total body weight of *E. diacanthus* has showed low correlation coefficient with the fecundity. Yashiro *et al.* (1993) have also observed the similar relation with total body weight and fecundity in *E. malabaricus*. It may be due to the fact that weight of the ripe gonads in relation to the total body weight of the fish is small.

Fecundity in *E. diacanthus* has showed linear relation with total length and standard length of the fish. It has shown low correlation coefficient r2 = 0.0217 and 0.01 respectively compared to the gonad weight (r2 = 0.5841). Tessy (1994) made similar observations in *E. diacanthus* and *E. bleekeri*. Bouain and Siau (1983) have also observed low coefficient of correlation with the fecundity and standard length in the grouper *E. aeneus*. However Chen and Hsieh (1980) found correlation with the standard length and fecundity in *E. diacanthus* from the Pacific Ocean.

In the present study, the average fecundity of *E. diacanthus* was 75,547. Highest fecundity recorded in the present study is 1,45,755. Tessy (1994) has reported that the average fecundity of *E. diacanthus* was 57,458 and the highest fecundity was 1,65,000. Chen and Hsieh (1980) have found that fecundity of E. diacanthus in the Pacific Ocean ranges from 63,000 to 2,33,000. Bouain and Siau (1983) have reported that for equal sizes (Standard length – 44cm) *E. aeneus* (Fecundity = 0.64 million) is more fecund than *Epinephelus guaza* (F = 0.60 million) and *E. alexandrinus* (F = 0.43 million). Estimates of potential fecundity in *E. tauvina* ranged from 0.85 million for a fish of 35.1cm long to 2.9 million for a fish of 62.3cm long (Abu-hakima, 1987). Selvaraj and RajaGopalan (1973) observed in one *E. tauvina*, the total potential fecundity estimated at 258.9 million.

The state of maturity of a fish may be determined by the size of ovaries. Gonado somatic index: (GSI) indicates the stage and readiness of the ovary for the maturation and spawning. Throughout maturation the GSI values in females were much higher than in males in *D. dentex* implying a greater proportion in body reserves were allocated in the gonads (Chatzifotis *et al.*, 2004). GSI has been used by many earlier investigators like Htun-Han (1978) to explain the degree of ripeness of ovary in a number of fishes. In the present study, the values of GSI for *E. diacanthus* has showed increasing trend from immature (0.06%) to ripe stage (3.06%). Yashiro *et al.* (1993) have also observed GSI values increasing from 0.43% to 5.2% with the maturation of gonads in *E. malabaricus*. The GSI values obtained in the present study correlated very well with the GSI values observed by Tessy (1994) in various size groups of *E. diacanthus*. Brule *et al.* (1999) have noticed the greatest variations in the mean gonado somatic index of female red grouper, *E. morio* from 0.27% to 2.14% in maturing and ripe running stages.

The liver provides accumulated energy reserves for reproductive growth and is also involved in the vitellogenesis of the fish oocytes, the relation of liver weight and weight of the fish expressed as HSI (Hepato Somatic Index). Krivobok (1964) has shown a direct correlation between liver weight and the size of oocytes in the Baltic Herring, *Clupea harengus membraneus* and also reported that females with heavier liver were more fecund than those with lighter ones. In the present study *E. diacanthus* HSI values increased from stage I (1.38%) to stage III (2.549%). Tessy (1994) has observed in *E. diacanthus* the HSI values were in the range of 1.1 to 3.1% in different size groups. The values reported in the above work are in agreement with the present study. Craig *et al.* (2000) have also reported the similar increasing trend in HSI values from 0.8% to 1.9% with the maturation of the gonads in the female red drum, *Sciaenops ocellatus*. Chatzifotis *et al.* (2004) have also observed HSI increased from F(2) to F(5) along with gonadal maturation in *D. dentex*.

The condition factor (K) and hepato somatic index are a measure of fish energy reserves. Conditional factor values follow inter annual variations and seasonal cycles (Lambert and Dutil, 1997). In the present study *E.diacanthus* condition factor values are in the range of 1.15 to 1.61. Condition factor has increased in *E. diacanthus* from stage I to stage III of gonadal maturation. Gopalakrishnan (1991) has reported the increase of condition factor with the advancement of maturation in *Mugil cephalus*. Hernandez *et al.* (2003) have also observed the increase of condition factor with the progress of reproductive season in the fish, *D. puntazzo*.

## 5.2 Histology and Ultrastructure of Oocytes and Hepatocytes

Liver produces vitellogenin after receiving estradiol stimulation from the ovary. Liver plays also a role in the synthesis of hormones. HSI increase of the liver, energy storage capacity of the hepatocytes and cytochemical characters of the hepatocytes depend on the physiological condition of the fish, feeding habits and nutrient availability (Svedong and Wickstorm, 1997).

In the immature stage , female hepatocytes of *E. diacanthus* have showed cytological characters of centrally located nucleus and cytochemical characters of scattered rough endoplasmic reticulum around the nucleus. Gopalakrishnan (1991) has also observed scattered endoplasmic reticulum in the immature female hepatocytes of *Mugil cephalus*. Similar cytological and cytochemical characters of hepatocytes of immature female fishes were also reported in the earlier works by Peute *et al.* (1978) in *Brachidanio rerio*, Bohemen *et al.* (1981) in *Salmo gairdneri* and Ribeiro *et al.* (2006) in *Steindachnerina insculpta*.

The maturing and ripe stage female hepatocytes of *E. diacanthus* have shown rapid proliferation of rough endoplasmic reticulum with flat cisternae and scant smooth endoplasmic reticulum. Enlarged mitochondria have also been

observed in the ripe female hepatocytes of *E. diacanthus*. The observations in the present study are agreeing with the earlier works reported in *S. gairdneri* (Bohemen *et al.*, 1981), in *Clupea harengus pullari* (Gillis *et al.*, 1990), in *M. cephalus* (Gopalakrishnan, 1991) and in *S. insculpta* (Ribeiro *et al.*, 2006).

Rapid proliferation of rough endoplasmic reticulum in mature female hepatocytes may be due to increasing vitellogenin (glycolipoprotein) production. Similar view has also been expressed by Peute *et al.* (1978) and Ribeiro *et al.* (2006). However, Bohemen *et al.* (1981) had detected no vitellogenin in the liver, even in the high lipid droplets suggesting that this protein is released into the blood stream immediately after its synthesis by the liver. Therefore increasing liver weight which resulted in increase of HSI with gonadal maturation may be related to the non-proteic substances and or development and proliferation of organelles in the cytoplasm of the hepatocytes during vitellogenesis (Bohemen *et al.*, 1981). According to Leatherland and Sonstegard (1988) presence of smooth endoplasmic reticulum may be due to the role played by the liver in metabolizing and converting sex hormones. Increase of density of cytoplasmic organelles in the mature female hepatocytes of *E. diacanthus* may be due to increasing energy demand and metabolic rate related to the maturation of gonads.

Oogenesis is the preparation for embryogenesis. It is characterized by the progressive accumulation of reserve materials used later in embryonic development. The storage of ribosomes during oogenesis is sufficient to ensure organogenesis and even cell differentiation. It is also clear that early germcells have been studied almost exclusively in fresh water teleosts mostly by light microscopy and only few accounts are available on marine species (Brusle and Brusle, 1978). By making electron microscopic studies, Brusle and co-workers have established the cytological criteria for the identification of early germcells by observing in the following species *Mugil auratus* (Brusle, 1980), *Epinephelus microdon* (Brusle-Sicard *et al.*, 1992), *Serranus hepatus* (Brusle, 1983) and *Amphiprion frenatus* (Brusle-Sicard *et al.*, 1994). They have reported cytological

criteria of high nucleus to cell ratio, abundant free ribosomes, a few mitochondria often forming association with nuages in the oocytes of immature, maturing and ripe female ovaries.

In the present study in female *E. diacanthus* with the progress of oogenesis from oogonia to perinucleolus stage, small nucleoli increased in number. Similar observations were also reported in *Barbus barbus* by Thiry and Poncin (2006). They have concluded that these small nucleoli could originate from the activation of some amplified rRNA genes. This indicates the activation of protein synthesis with the progress of oogenesis.

In the present study in *E. diacanthus* maturing oocytes have showed thin zona radiata development in the process of oogonial development. Gopalakrishnan (1991) also made similar observations in *M. cephalus*. In ripe female *E. diacanthus* vitellogenic oocytes have contained the following cytological and cytochemical characters. The increase of dense rough endoplasmic reticulum, movement of germinal vesicle towards the periphery, yolk globules formation, development of thick zona radiata, zonation in yolk globules and mitochondrial aggregation. Goplakrishnan (1991) has observed the increased intensity in rough endoplasmic reticulum, Yolk globule presence in the cytoplasm and thick zona radiata in the vitellogenic oocytes of *M. cephalus*. The observations made in the above study are similar to the cytochemical and cytological characters noticed in the vitellogenic oocytes of *E. diacanthus*. Lal (1991) has also made similar observations in the vitellogenic oocytes of *Lates calcarifer*.

Yolk globules in vitellogenic oocytes of *E. diacanthus* have showed zonation of electron dense inner region and electron lighter outer region. It indicates transition of yolk spheres in the penultimate stage of vitellogenesis. Lal (1991) has also observed similar zonation of yolk globule in *L. calcarifer*.

However in *M. cephalus*, yolk globules did not show zonation (Gopalakrishnan, 1991).

## 5.3 Hermaphroditism

Hermaphroditism and gonadal sex inversion is a normal mode of reproduction in a number of serranids (Atz,1964). As suggested by Smith (1965) groupers reproductive mode is usually of Epinephelus type in which the entire gonad is an admixture of ovarian and testicular tissues. He also pointed that protogynous mode of reproduction is common in a number of groupers in the family Epinephelinae. In the *E. diacanthus* gonads, the histological observations have revealed the presence of Epinephelus type and protogynous mode of reproduction. Tessy (1994) has also found similar histological observations in *E. diacanthus*.

Histological observations of *E.diacanthus* ovary have shown the regressing oocytes with the proliferation of spermatogenic crypts. It indicates the presence of protogynous mode of reproduction in *E.diacanthus*. Brusle *et al* (1989) have noticed the seminiferous crypts scattered in the parietal part of ovarian lamella in protogynous hermaphroditic fish *E. microdon.* Some investigators believed that the identification of degenerative process inside the gonadal tissue reveals sex inversion (Moe, 1969; Abu-Hakima, 1987). In the present study much effort was not taken to study related aspects of physiology in relation to sex inversion. The work was mainly oriented to find out the main links in the reproductive physiology of female *E. diacanthus*.

## 5.4 Electrophoretic isolation and characterization of vitellogenin

Vitellogenin is a glycolipoprotein that is produced by liver in response to circulating estrogen, released into the blood stream, taken up by the developing oocytes and chemically modified in the process of yolk formation. Because vitellogenin deposits from the principal nutritive reserve of eggs and constitute

the major food supply of embryos before external feeding, knowledge of the biochemistry of fish vitellogenin will improve our understanding of fish reproduction and nutritional requirements of larvae.

In the present study *E. diacanthus* yolk protein was isolated and characterised by the gel electrophoresis. The results were duly supplemented with special staining of protein bands in native PAGE. In the present study native PAGE run of ovarian homogenates and serum samples of female *E. diacanthus* with different maturity stages have showed the increase of intensity of high molecular weight protein bands with the maturation of gonads. The protein bands in ripe female serum and ovarian homogenate of *E. diacanthus* were more prominent than the immature, maturing and male gondal tissue homogenate. *E. diacanthus* protein bands expressed in the gel electrophoretic run has shown specificity for the gender and the maturity stage of gonads. Similar results were obtained by Bochard (1968) who observed that high molecular weight protein fractions increase with the maturation of gonads in the female rainbow trout (*Salmo gairdneri*). In the ultrastructural studies of the present study, the increase of dense endoplasmic reticulum at cellular levels of oocytes and hepatocytes substantiated the increase of vitellogenin synthesis with the maturation of gonads. The vitellogenin synthesized in the target organ was transported to the oocytes by serum. The abundance of serum vitellogenin protein bands in the ripe female serum is correlated with the above results.

The intensity of prominent protein bands with the maturation of gonads was also observed in female grey mullet, *Mugil cephalus* (Gopalakrishnan, 1991). It has been reported that high molecular weight protein bands were prominent in *E. malabaricus* whereas these bands were absent in the juveniles (Utarabhand and Bunlipatanon, 1996). Increase of vitellogenin specific protein bands with the estradiol inducement was observed in the ocean pout (*Macrozoarces americanus*), lump fish (*Cyclopteus lumpus*) and Atlantic cod (*Gadus morhua*) (Yao and Crim, 1996).

*E. diacanthus* mature male gonadal tissue homogenate protein banding patterns are not as prominent as ripe female ovarian homogenate. Copeland *et al.* (1986) have also observed low vitellogenin levels in the male rainbow trout, *Salmo gairdneri*. However, Borchard (1968) had seen the presence of high molecular weight fractions even with matured male.

Native *E. diacanthus* vitellogenin exists in two heterogenous molecular weight forms (MW = 583.6kDa and MW = 407.5kDa) and after denaturation 583.7kDa molecular weight form has expressed two sub units (MW= 262.6kDa and MW= 321.0kDa). The results may be explained by the fact that in ripe stage the high molecular weight proteins as mentioned earlier have been synthesized from lower molecular weight precursors. This hypothesis is further supported by the SDS gel electrophoretic pattern where lower molecular weight protein sub units 89.69 kDa, 62.34 kDa, 40.33 kDa, 29.08 kDa, 20.67 kDa and 17.45 kDa are not present in the ripe serum sample. The band at 583.6 kDa as appeared in native PAGE is a dimeric protein which under denaturing condition in SDS gel electrophoresis has been bifurcated into two smaller subunits appeared at 262.6kDa and 321.0kDa. Other protein bands at 111.8 kDa, 93.8 kDa, 58.9kDa and 29.6 kDa appeared to be very less prominent in the ripe serum sample thus allowing us to conclude that these smaller molecular weight protein bands may function as precursor biomolecules for larger molecular weight protein. No prominent bands were observed in the immature serum. The earlier results indicate that proteins appearing at this high molecular weight in the ripe stage of female gonadal maturation belong to vitellogenin family proteins. The above results are also confirmed by the biochemical characterization of these proteins through the special staining for glycolipoprotein with calcium moiety. Utarabhand and Bunlipatanon (1996) noticed vitellogenin as a high molecular weight dimeric form (MW = 525kDa) in *E. malabaricus*. Similar observations in the vitellogenin subunits were reported by de Vlaming *et al.* (1980) in gold fish (*Carassius auratus*) and Hara *et al.* (1980) in Japanese eel (*Anguilla japonica*).

*E. diacanthus* ripe female serum and ovarian homogenate have indicated the character of glycolipoproteins with calcium moiety. Glycolipoproteins are the main source of amino acids and lipids for the embryonic development to which calcium is ionically bound. Thus glycolipoproteins with calcium moiety delivers the minerals needed by the developing embryos for skeletal development and metabolic functions (Patino and Sullivan, 2002). In the present study intense bands have appeared for calcium staining in the ripe females as compared to the immature and maturing samples. Gopalakrishnan (1991) had also reported similar observations in female *Mugil cephalus* with the maturation of gonads.

Vitellogenin protein markers of *E. diacanthus* as developed in the present study are unique and novel in this species of groupers which can be utilized for the identification of gender and reproductive maturity of the animal. The information generated in the present study is useful for developing immunological products which can be helpful in the initiation of maturation. Even though much work was carried out on the sex reversal and induced spawning of groupers we failed to standardize the grouper hatchery technology. High larval mortalities were reported in the earlier attempts. Biochemical characterization of the vitellogenin as in the present study with further studies by using chromatographic techniques and profiling amino acids and fatty acids will give solutions to the bottlenecks in the larval survival and hatchery technology development of groupers.

## 5.5. Biochemical composition

Reproduction is a dynamic metabolic activity. Maturation of gonad involves complex physiological changes accompanied by the profound changes in the chemistry of fish. The macronutrients such as protein, lipids, cholesterol, carbohydrates, minerals etc., undergo variation during the process of gonadal

maturation to provide complete nourishment for the developing eggs. Amino acid accumulation for germ cells production marks the deposit of proteins in the developing gonads. Fat accumulated during active feeding stage is utilized by the fish during spawning migration. In the early stages of maturation, appetite increases, resulting in the accumulation of fat depots. The energy needed for gonadal development during the reproduction in the animal is provided by the energy reserves in the fish (Love, 1980).

Shearer (1994) reviewed the factors that affect fish composition and physiological changes. Factors can be either endogenous or exogenous and some times they operate together. The endogenous factors are genetically controlled and are associated with the life cycle of the animal. As the gonads increase in size, somatic growth slows down and eventually stops. At this time proteins and lipids are mobilized from the muscle and transferred to the gonads (Aksnes *et al.*, 1986).

Carbohydrate role cannot be denied in the immediate energy supply. Glycogen and glucose have both been reported to accumulate in the gonad, especially in the females (Chang and Idler, 1960). Cholesterol showing minimal value during greatest sexual activity is found associated with the formation of sex hormones. Carotenoids are attributed with the function of maintaining egg quality, imparting colorations and controlling the viability of eggs.

The spawning and spermiation in common dentex (*D. dentex*) has been demonstrated to evince major alterations in the blood chemistry along with tissue composition changes (Chatzifotis *et al.*, 2004).

**Moisture**

Moisture plays a decisive role in several biochemical functions, because of its ionic properties and its association with other constituents. Its fluctuations in

the body tissues, besides being influenced by environmental factors, osmotic properties of the cells and other profound physiological activities, serve as an indicator of accumulation or drain of metabolic components in tissues or cells (Elizabeth, 1987; Gopalakrishnan, 1991 and Lal, 1991).

In the present study, it is observed that in *E. diacanthus*, moisture percentage decreased in the ovary, liver and muscle with the maturation of gonads. Ovary moisture percentage is high in immature stage. It may be concluded that reduced moisture in stage III was replaced with proteins, lipids and other nutrients as the gonadal development progresses. In the present study it is observed that protein, lipid, cholesterol, carotenoids and minerals of ovaries increased with the maturation of gonads. It was observed that there is inverse relation with the fat and moisture and also protein and moisture. Hernandez *et al* (2003) had observed similar pattern of relation with fat and moisture contents associated with growth periods and sexual maturation in *Diplodus puntazzo*. Sivakami *et al* (1986) also found that muscle moisture percentage decreased from stage II of female gonadal maturation on wards in the *Cyprinus carpio*. Muthukaruppan (1987) and Gopalakrishnan (1991) observed the similar trend in *Liza parsia* and *Mugil cephalus* respectively.

With the advancement of maturation major energy reserves in the muscle are mobilized for the growth of gonads. Masurekar and Pai(1979) had seen the fluctuating muscle water content during the maturation of gonads in *Cyprinus carpio*.

**Proteins**

Reproduction involves high energy metabolic activity, including germ cells production and maturation. Germ cell production demands high protein *requirement*. The proteins required for ovarian development are met by exogenous or endogenous factors. Endogenous factors involve thorough

mobilization of energy reserves from one tissue to the other tissues and exogenous through feed intake. During the process of maturation, fishes which undergo starvation totally dependent on body tissue reserves. Energy requirements for germ building require large quantities of amino acids. Proteins play a vital role in somatic and reproductive growth of the fish. Protein composition of the fish is subject to variations depending upon the season, feed intake, breeding and migration.

*E. diacanthus* muscle protein percentage increased with gonadal maturation. Muthukaruppan (1987) also observed muscle protein content increase from stage I to stage III of *Liza parsia* during gonadal maturation. Ando and Hatano (1986) studied biochemical characteristics of Chum salmon muscle during spawning migration and they found a good positive correlation between gonado somatic index and muscle protein percentage increase of female fish. Sivakami *et al.* (1986) had found that muscle protein percentage increased form the second stage of gonadal maturation.

However Masrurekar and Pai (1979) had observed the decrease of muscle protein with the maturation of gonads in *Cyprinus carpio*. They have concluded that muscle protein might have been mobilized to meet the demand of gonads in sexual reproduction.

Even in some teleost fishes which undergo starvation during the spawning it has been demonstrated that depletion of muscle and liver protein and the mobilization of these to the gonads is a routine act. But depletion may not be reflected immediately in terms of muscle and liver protein alone.

In the present study *E. diacanthus* liver protein percentage increased from immature stage to ripe stage. Liver is the dynamic site for the synthesis of hormones and energy reserves which are mobilized for the development of gonads in sexual reproduction. In the present study moisture content of the liver

decreased from immature stage (stage I) to ripe ovary stage (stage III). It may be concluded that proteins have replaced the moisture drained space of liver.

*Epinephelus diacanthus* liver protein percentage has increased from stage I to stage III. Liver synthesizing proteins during maturation has already been proved histologically in Ayu fish (Aida *et al.*, 1973).

In the present study ovary protein percentage increased with the maturation of gonads in *E. diacanthus*. Robards *el al.* (1999) had found protein percentage of gonad increased in the pacific sand lance in relation to maturity. In *Mugil cephalus* , Gopalakrishnan (1991) observed ovary protein percentage increased with the maturation of gonads. Muthukaruppan (1987) also observed the same trend in *Liza parsia*.

Blood serum plays a major role in the maturation of gonads. In the present study, serum protein percentage showed a gradual increase with the progress of maturation. Chatzifotis *et al.* (2004) found that serum protein percentage increased with the maturation of gonads in the common dentex (*Dentex dentex*).

The study did not exhibit any subsequent decrease of body tissue proteins content with the ovary protein increase. Hence it may be inferred that exogenous factors such as food in take was the major source for protein percentage increase of gonads. Tessy (1994) has reported the protein rich food intake increased from immature to ripe fishes in *E. diacanthus*.

**Carbohydrates**

Muscle carbohydrate content pattern in the present study showed subsequent fall and rise from stage I to stage III. Muthukaruppan (1987) also observed the same trend in *Liza parsia*. It is discerned that carbohydrates

content decreased with the translocation of carbohydrates from depot site to where the energy prompt is required. However, Sivakami *et al.* (1986) observed gradual increase of muscle carbohydrate content with the maturation of gonads in the *C. carpio*.

In the present study *E. diacanthus* liver carbohydrate percentage has showed high fluctuations with the maturation of gonads. Liver is the major site for carbohydrate reserves. It is used up continuously for energy requirements in the activities including the gonadal development. To cope up with these requirement prompt translocation of reserved energy should take place from body tissues.

In the present study ovary carbohydrate percentage of *E. diacanthus* increased from stage II to stage III. Such results were observed earlier by Sivakami *et al.* (1986), Muthukaruppan (1987), Gopalakrishnan (1991) and Lal ( 1991) for *Cyprinus carpio*, *Liza parsia*, *Mugil cephalus* and *Lates calcarifer* respectively.

*E. diacanthus* serum carbohydrates have showed a pattern quite opposite to the trends observed in muscle, ovary and liver. They first increased from stage I to stage II and later decreased in the stage III. It indicates that serum played a role of transporting agent of carbohydrates from body tissues to reproductive organs.

Chatzifotis *et al.*, (2004) have observed that serum glucose concentration in common dentex decreased from stage I to stage II and as subsequent stages it increased. It may be due to environmental conditions and feed in take of fishes during the season.

Carbohydrate content high fluctuation discerned that where energy is immediately required, glycogenolysis progressed and carbohydrates met the

energy demand. As the animal progressed further in maturation the usual feed intake might have balanced the demand for somatic and reproductive growth.

**Lipids**

Vertebrates use their lipid reserves for various needs such as growth, maintenance and reproduction. Seasonal variations in the stored lipid values have been observed in many fish species. Nelson and Mc Pherson (1987) had observed decrease in lipid content in muscle and viscera of Brook char (*Salvelinus fontinalis*) with the progress of reproduction. Fats have high calorific value and are stored in muscle, liver, intestine and gonads. In the spawning season, the fish draw upon these stores for the growth and development of reproductive elements. In the present study it has been observed that muscle lipid content of *E. diacanthus* got depleted with the maturation of gonads. Masurekar and Pai (1979) also observed muscle fat content depletion with the maturation of gonads in the *Cyprinus carpio*.

Earlier works on *Sparus auratus* (El-sayed *et al.*, 1984), in pelagic sculpins (Kozlova, 1997), in Chum salmon (Ando and Hatano, 1986) and in *Liza parsia* (Muthukaruppan, 1987) also a correlation of the muscle fat depletion with the maturation of gonads was reported.

However, it has been observed that muscle lipid content increased with the maturation of gonads in *Cyprinus carpio* ( Sivakami *et al.*,1986). Common dentex muscle lipid content also showed the same trend with the maturation of gonads ( Chatzifotis *et al.*, 2004).

In the present study, liver lipid percentage has decreased with the development of gonads. El-sayed et al (1984) had observed minimum liver total lipid content with the progress of gonadal maturation in the *Tilapia nilotica* and *Sparus auratus*. Liver is a major biosynthetic and depot organ for lipids. Lipid

reserves are utilized for the growth of reproductive organs with the maturation of ovary. Kozlova (1997) had reported the utilization of lipids in the liver of pelagic sculpins for the gonadal development. He had observed the subsequent depletion of liver lipids with the maturation of gonads. The total lipid content of the liver of steel head trout was observed to have decreased with the maturation of gonads (Sheridan *et al.* 1983). In the present study total lipid content of the liver has decreased with the maturation of gonads. Same trend in liver lipid content was observed in *Liza parsia* and *Mugil cephalus* with the maturation of gonads (Muthukaruppan, 1987 and Goplakrishnan, 1991)

Lowering of liver lipid levels were noticed in red drum, *Sciaenops ocellatus* with the gonadal maturation (Craig *et al.* 2000). In common dentex (*D. dentex*) a rise and fall of liver lipid levels with the maturation of gonads was noticed (Chatzifotis *et al.* 2004). Depletion of the liver total lipid content with the maturation of female germ cells was observed in the protandrous hermaphrodite *Lates calcarifer* (Lal, 1991). In the present study on *E. diacanthus*, total lipid content of the liver has showed a similar trend as observed in earlier works on other fishes. In the present study results indicate that the liver plays the role of energy reserves for the reproductive growth of female germ cells in *E. diacanthus*.

Serum plays the role of transporting agent of nutrients with the maturation of gonads. In the present study serum total lipids increased from stage I to stage III. Chatzifotis *el al.* (2004) correlated the total serum lipid content increase with the maturation of gonads in common dentex. Similar increase in serum total lipid levels were observed with the *Lates calcarifer* also ( Lal, 1991).

Much of yolk material of the egg of teleost fishes generally contained a lipophosphopoprotein complex. Mobilization of lipids takes place during the spawning period to the gonads. In the present study it was observed that the lipid content of ovary increased drastically from stage I to stage III. El-sayed *et al.*

(1984) had seen the similar trend in lipid content change in *Tilapia nilotica* and *Sparus auratus*. Sivakami et al., (1986) had observed that the lipid levels increased in the ovary of *Cyprinus carpio* with the maturation of gonads. Muthukaruppan (1987) in *Liza parsia* and Gopalakrishnan(1991) in *Mugil cephalus* have also observed similar trends in ovary lipid with the progress of gonadal development.

Lal (1991) had observed the correlation in the lipid content in ovary with the maturation of gonads in *Lates calcarifer* and found that lipid content increased with the maturation of female from stage I to stage IV. However, Chatzifotis *el al.* (2004) have noticed in common dentex(*D. dentex*) the decrease of ovary lipid content from stage I to stage II and subsequent increase in the process of gonadal development.

In the present study it was noticed that the correlation in the lipid content in different body tissues of *E. diacanthus* varied with the maturation of gonads. It was observed that liver lipid content drastically decreased from stage I to stage III and that consequently ovary lipid content increased with maturation of gonads. This is suggestive of the mobilization of liver and muscle lipids to the ovary with the progress of vitellogenesis.

**Cholesterol**

Cholesterol plays the major role in the synthesis of steroids, which in turn influence the maturation phenomena. In the present study it was observed that the cholesterol levels decreased in the muscle with the maturation of gonads. It can be inferred that muscle cholesterol played the role in steroid synthesis.

Liver cholesterol levels of *E. diacanthus* decreased with the maturation of gonads. Lal (1991) also observed same trend in liver cholesterol with the

maturation of female germ cells in *Lates calcarifer*. Low levels of cholesterol in liver may be due to higher physiological needs of gonadal development.

In the present study cholesterol content of the ovary was observed to decrease from stage I to stage III. Cholesterol level depletion has indicated the progress of steroid synthesis as the gonadal maturation reaches final stage. Lal (1991) also observed similar trend in the ovary cholesterol content changes with the maturation of female gonads in *Lates calcarifer*. Jayashree and Srinivasachar(1979) had noticed in *Clarias batrachus* the lowest level of cholesterol in the ripe male.

However Diwan and Krishnan (1986) observed fluctuation in the cholesterol levels of the gonads with the maturation of gonads in *Etroplus suratensis*. In immature female fish gonadal cholesterol was high and as maturation progressed in subsequent stages cholesterol level had showed fall and rise. They have concluded that steroid synthesis progressed with the gonadal development leading to fluctuations in gonadal cholesterol levels.

Serum is the transporting medium for the steroids to reach the target organs. In the present study serum cholesterol levels decreased from stage I to stage II. Highest cholesterol level was recorded in stage III. Lal (1991) had observed the same trend in serum cholesterol levels with the maturation of female germ cells in the *Lates calcarifer*. Chatzifotis *et al.* (2004) also observed serum cholesterol increase from stage I to stage IV of the female common dentex. Furrel and Muni (1983) also reported that serum cholesterol level elevation with the spawning in Atlantic Salmonids.

**Carotenoids**

Carotenoid pigment levels in the muscle, liver and ovary are determined both by the pigment content of the feed and uptake efficiency which in turn is dependent on the carotenoid requirement of each species. In *E. diacanthus* muscle carotenoids increased with the maturation of gonads. A gradual increase of carotenoids content with the maturation of gonads was noticed

However, Kitahara (1983) has observed that chum salmon muscle carotenoid content decreased with the maturation of gonads. He even noticed mobilization of muscle carotenoids to the skin. Crozier (1970) detected that the high carotenoid content of the sock eye salmon muscle decreased with the progress of maturation of gonads. It may be concluded that the carotenoids content increase *E. diacanthus* is depended on feed in take. Groupers consuming large amounts of crustaceans such as crabs (which are rich in carotenoids) has been reported (Tessy, 1994).

Liver carotenoid content increased from stage I to III of gonadal maturation in *E. diacanthus*. It is noticed that carotenoids concentration in the liver is high compared to ovary, muscle and serum. According to Leger (1985) liver is the site of synthesis of lipoprotein. Liver plays the major role in mobilization of carotenoids to the ovary with the maturation of gonads. Patnaik (2001) had found that in *Priacanthus hamrur* carotenoid content of liver increased from stage I to stage II of gonadal maturation and it decreased in the subsequent stages up to stage VI.

*E. diacanthus* ovary carotenoids have increased from stage I to stage III. During sexual maturation considerable amount of carotenoids are transferred to the yolk (No and Storebakken,1992). According to Bjerking and No (1992) the

carotenoid content in the female gonad increases moderately with the maturation. In the present study *E. diacanthus* ovary carotenoid content increased from stage I to stage III. Patnaik (2001) had noticed the increase in carotenoid content of the ovary of *P. hamrur* with the maturation of gonads.

Serum carotenoid content increases during on set of vitellogenesis. In the present study ovary carotenoid content increased with the maturation of gonads. Highest serum carotenoids are noticed in the stage III of gonadal development. It indicates that the carotenoids are transported by serum to the developing eggs in the ovary. Plack and Woodhead (1966) also reported the increase of carotenoid compounds in the serum of ripe female cod.

**Minerals**

Fishes get most of the minerals required for germ cell production through osmosis, but less minerals mobilization for gonadal development will take place. In the present study minerals content decreased in the muscle from stage I to stage III, but consequential mineral content increased in the ovary was also noticed from stage I to stage III. It indicates that minerals mobilization from muscle to ovary has taken place with the maturation of gonads. Sivakami *et al.* (1986) had observed decline of muscle ash content from stage III to stage V in the female *Cyprinus carpio.*

Liver mineral content of *E. diacanthus* has also decreased from the stage I to stage III of gonadal maturation. This decrease in mineral content may be explained as ;mobilization of minerals required for gonad buildup in female *E. diacanthus* is evidently transferred from muscle and liver to the developing ovary.

# *Summary*

# SUMMARY

The present study was aimed at understanding the detailed morphological, cytological and ultratructural changes occurring in the ovarian tissue during the different stages of maturation, and also the detailed biochemical changes happening in major tissues of the female grouper, *E. diacanthus*.

The study was carried out from collections of the fish onboard FSI vessel during the cruises off Quilon and off Ratnagiri region. The study was carried out at CMFRI, Cochin from September 2002 to August 2004.

A brief introduction is given in the beginning of the work to explain

a) The need of an in-depth understanding of the reproductive physiology of groupers in the mastery of controlled seed production
b) The paucity of information related to reproductive physiology of Indian groupers and
c) Substantiating the purpose of the study in the smaller sized *E. diacanthus* sustaining one of the major demersal fisheries of India.

A detailed review of literature covering various aspects of work on food fishes and groupers on specific themes such as oogenesis, ultra structural studies, hermaphroditism, sex reversal, vitellogenesis, vitellogenin isolation and characterization and biochemical composition has been made.

A detailed material and methods section is given describing the selected species, sites of collection, collection and preservation of samples and also detailed methodology of collection of biological data such as oocyte size, GSI (Gonado Somatic Index) , HSI(Hepato Somatic index), condition factor and fecundity. The methodology adopted in histological studies both for light microscopy and ultra structural studies with details of processing of samples is also described. The methodologies followed for isolation and characterization of vitellogenin is described in detail. Procedures for the estimation of

biochemical parameters of major tissues such as ovary, liver, body tissues and blood serum are also elaborated.

The results obtained in the various lines of investigation are elaborated in separate sections. The morphological details of female reproductive system of *E. diacanthus* are described in detail. A morphological classification of maturity stages detailed as stage I, II and III is also described.

The distribution of ova in the ovary in the three stages and their major oocyte size frequency is elaborated. In the stage I of maturity the dominant oocyte size frequency noticed was 0-50µm. ; In the stage II ovary 201 - 250 µm was the major oocyte size frequency and in stage III it was 501 - 550 µm. Maximum oocyte size of 650 µm was observed in the present study. The Gonado Somatic Index of *E. diacanthus* is described in detail showing a positive correlation with the maturation of gonads. Highest GSI values observed was in stage III ovary (3.064%).The Hepato Somatic index of *E. diacanthus* was also worked out, the values showing a maximum in stage III of gonadal maturation (2.549%). The HSI values were showing positive correlation with the maturity stages. The condition factor 'k' was worked out for *E. diacanthus*. The values were in the range of 1.15 – 1.61.The fecundity of *E. diacanthus* was determined. The fecundity was observed to vary from 13.1 X $10^3$ to 145.7 X $10^3$ with an average of 75, 547.

The relation between fecundity and weight of ovary, which was linear also was found out for *E. diacanthus* ($r^2$ = o.5841). The relationship between fecundity and total weight was worked out and the relationship proved out to be linear showing the gradual increase of fecundity with total weight.

The relationship between fecundity and total length was assessed and it showed low correlation coefficient (r2 = 0.0217). The relationship between fecundity and standard length was also worked out and the relationship proved to be least significant ($r^2$ = 0.01) Detailed microscopic studies of the different stages of maturity of female *E. diacanthus* was done and three stages 1) Immature 2) Maturing and 3) Mature / Ripe were observed and these were

studied elaborately describing the cellular changes in depth. Ultra structural studies to understand cytological details of oocytes in the three maturity stages were also conducted. The data on details of cytological and cytochemical changes such as increase of density of cell organelles such as mitochondria, endoplasmic reticulum, golgi bodies associated with the maturation of gonads were collected.

The dense endoplasmic reticulum, mitochondrial aggregation and presence of mitochondria with tubular cristae were observed and explained in the mature ovary which were less dense or absent in the immature stages. The well developed Zona radiata was observed in the mature ovary. The zonation in the yolk globules was also observed.

A similar ultra structural study of hepatocytes describing cytological and cytochemical changes associated with gonadal maturation were elaborated. Dense endoplasmic reticulum with flat cisternae in the hepatocytes was decribed in the mature stage of ovary. The transitional stage of gonads in *E. diacanthus* was explained from microscopic sections. The development of spermatocytes and degeneration of oocytes occurring in such transitional gonads were explained in detail showing the protogynous mode of reproduction.

Native PAGE of gonadal tissue homogenate and serum samples of different maturity stages was conducted to resolve the level of expression of yolk protein during the maturation of gonads. The protein bands of ripe female ovarian homogenate are more prominent among all the protein bands as expressed in other maturity stages and male gonads.

Ripe female protein bands as expressed in the native PAGE exhibited its glycolipoprotein with calcium moiety. The first protein band ($Rf_1$ = 0.155) appeared to be more distinct as compared to those of male and female tissue homogenates of other stages.

The ripe female serum protein bands 6 and 7 expressed positive staining with Periodic acid schiff's reagent, Sudan black B and Alizarin red. Native vitellogenin was described existing in two heterogeneous molecular weight

forms( MW=583.6 kDa and MW = 407.5 kDa) and after denaturation 583.7 kDa molecular weight form has expressed two sub units (MW = 262.6 kDa and MW = 321.0 kDa).

To elaborate the biochemical changes during maturation the biochemical composition of four tissues namely ovary, liver, body tissue and blood serum were studied. Seven major parameters such as proteins, carbohydrates, lipids, cholesterol, carotenoids, moisture and ash were estimated in the four tissues in relation to maturation of gonads.

In muscle, moisture content, cholesterol, lipids and ash percentage decreased with the maturation of gonads. Carbohydrate percentage showed fluctuations of increase and decrease with the maturation of gonads. Protein percentage and carotenoids level increased with the maturation of gonads.

The protein percentage in the liver was observed from stage I to stage III. The maximum liver protein percentage was noticed in ripe stage (22.30%). Liver carotenoids content showed positive correlation with the maturation of gonads. Highest carotenoids content was observed in the mature stage (11.3 µg /g). Carbohydrate percentage showed fluctuations with the maturation of gonads. Moisture percentage of liver decreased from stage I to stage III. Liver ash percentage was in the range of 0.39 to 0.81%. Ash percentage of liver also decreased with the maturation of gonads, which indicated the mobilization of minerals to the reproductive organs with the progress of maturation. Liver lipid percentage showed decreasing trend with the maturation of gonads.

Blood serum protein percentage increased from stage I (6.61%) to stage III (10.98%). The cholesterol percentage showed fluctuations with the maturation of gonads. Total carbohydrate percentage also showed fluctuations with the maturation of gonads. Serum carotenoid levels were low compared to liver, body and ovary. In stage I of gonadal maturation the value was 0.17µg / g and it rose to 0.95 µg / g in stage III.

Ovarian moisture percentage showed inverse relationship showing a steady decrease from stage I (71.66%) to stage III (60.58%). The protein percentage in the ovary was observed having a positive relation with the maturation showing an increasing trend. The highest protein percentage was noticed in stage III( 24.81%). The total carbohydrate content showed a fluctuating trend. The total lipid percentage of the ovary showed a positive relationship by a gradual increase from stage I (6.56%) to stage III (13.48%). Cholesterol levels of the ovary showed a steady declining trend with the maturation of gonads. Carotenoid levels of the ovary showed a positive relationship. Ovary ash percentage increased from stage I (0.82%) to stage III (1.20%).

The biochemical composition results obtained in the present study showed significance at 1% level and 5% level when subjected to statistical analysis.

A detaliled discussion on the observations arrived at through the study is made and efforts to compare these results with other similar works has also been attempted.

Throughout the discussion a stress on the need of conducting more studies in the same lines is made to help us in the mastery of controlled reproduction of groupers

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* Not referred in original

# *Appendices*

# Annexure-1

Acetone gradient used in Transmission Electron Microscopy

1. 30% - Two changes of 20min. each
2. 50% - Two changes of 20min. each
3. 70% - Two changes of 20min. each
4. 80% - Two changes of 20min. each
5. 85% - Two changes of 20min. each
6. 90% - Two changes of 20min. each
7. 95% - Two changes of 20min. each
8. Absolute Acetone - Two changes of 20min. each